Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 64 FEBRUARY, 1947 No. 2

SECTION MEETINGS

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15723

A Fat-Soluble Material from Plasma Having the Biological Activities of Biotin.

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It is now well established that biotin deficiency decreases the resistance of chickens and ducks to malaria parasites.[1,2] It also appears to decrease the resistance of rats to *Trypanosoma lewisi*[3] and of mice to mouse typhoid.[4] A normal level of biotin must therefore be essential to the proper functioning of some general mechanism of resistance to infection. In the course of work directed toward discovering the nature of this resistance mechanism, it was found that the plasma of a variety of animals contains a previously undescribed material which, after hydrolysis by acid or enzymes, yields a fat-soluble substance having the biological activities of biotin but differing from it chemically. This material is probably more intimately concerned in resistance to malaria parasites than is biotin itself. The changes in concentration which both it and biotin undergo in the plasma of chickens and ducks infected with malaria, and its effects on the multiplication of malaria parasites *in vitro* will be described elsewhere. It is the purpose of the present paper to present the data demonstrating the existence of the material and to relate some of its biological and chemical properties.

Of the various methods of microbiological assay for biotin, one of the most specific is that employing *Lactobacillus casei*.[5] The *L. casei* assay has therefore been used almost exclusively. The medium and method of in-

1 Trager, W., *J. Exp. Med.*, 1943, **77**, 557.

2 Seeler, A. O., Ott, W. H., and Gundel, M. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 107.

3 Caldwell, F. E., and György, P., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 116.

4 Kligler, I. J., Guggenheim, K., and Herrnheiser, H., *J. Infect. Dis.*, 1946, **78**, 60.

5 Shull, G. M., Hutchings, B. L., and Peterson, W. H., *J. Biol. Chem.*, 1942, **142**, 913.

6 Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, 1942, **27**, 1086.

7 Skeggs, H. R., and Wright, L. D., *J. Biol. Chem.*, 1944, **156**, 21.

TABLE I.
Biotin Activity of Human and Duck Plasmas After Different Types of Treatment.

Plasma	Treatment	Activity as mγ biotin per ml plasma
Human	(1) Diluted in water.	2.0
	(2) Mixed with 10 X vol. of 3 N H_2SO_4, autoclaved one hr, then neutralized.	11.4
	(3) Precipitated with trichloracetic acid. Autoclaved 1 hr.	9.7
	(4) Precipitated with trichloracetic acid. Precipitate removed by centrifugation. Supernatant autoclaved 1 hr.	1.8
Duck A	(1) Diluted in sterile water. Added aseptically to assay tubes.	3.2
	(2) Autoclaved 1 hr in 10 X vol. of 3 N H_2SO_4. Neutralized.	14.6
	(3) Same as (2) but filtered before neutralization.	3.6
	(4) 24-hr treatment with takadiastase in acetate buffer, pH 4.7. Then neutralized and autoclaved.	15.7
Duck B	(1) Diluted in buffer, pH 8.0.	4.2
	(2) Same as (1) followed by ether extraction.	2.5
	(3) Autoclaved 1 hr in 10 X vol. of 3 N H_2SO_4. Brought to pH 8.2.	10.0
	(4) Same as (3) followed by ether extraction.	1.3
	(5) 24-hr treatment with takadiastase in acetate buffer, pH 4.7. Then neutralized and autoclaved.	9.8
	(6) Same as (5) followed by ether extraction.	2.3

oculation were those described by Landy and Dicken[6] except that the vitamins of the B complex were added in larger amounts,[7] pure crystalline folic acid (obtained from Lederle Laboratories through the courtesy of Dr. Y. SubbaRow) was used at 25 γ per l of double-strength medium and pyridoxamine (Merck) was added at 10 γ per l. Growth was determined by titrating the acid produced with 0.1 N sodium hydroxide. Since it soon became apparent that a substance other than biotin was being measured in terms of its biotin activity, it was found advantageous to prolong the time of incubation of the assay tubes from the usual 3 days to 4. When this was done, the results obtained with 3 different concentrations of the same sample usually agreed within 10 to 15%.

Acid hydrolysis of plasma was accomplished by mixing 0.4 ml of plasma with 4 ml of 3 N sulfuric acid and autoclaving at 15 lbs for one hour, a procedure designed to liberate bound biotin.[8] Plasma was also hydrolyzed by treatment with takadiastase,[9] 5 mg of enzyme being used to 0.5 ml of plasma diluted to 5 ml with acetate buffer of pH 4.7.

It was found that if normal plasma was hydrolyzed with sulfuric acid and filtered, either before or after neutralization, its biotin content was the same as that of plasma merely diluted in water, indicating that plasma does not contain any bound biotin. But if the hydrolyzed plasma was not filtered, a much higher biotin activity was found. Similar results were obtained after hydrolysis by takadiastase. All of the additional biotin activity resulting from the hydrolysis of plasma could be removed by shaking with ether, as well as by filtration. The shaking of unhydrolyzed plasma with ether removed a variable proportion of its already low biotin activity but did not affect the activity which appeared upon subsequent hydrolysis. Table I illustrates a few typical results out of many which have been obtained. In one series 6 mγ of pure biotin were added to duplicate 0.4 ml samples of plasma. These, and the corresponding samples without added biotin were submitted to various treatments and assayed for biotin. The % recovery of the added biotin was: 97 for the samples diluted in water, 89 for the samples autoclaved in sulfuric acid, brought to pH 7 and not

[8] Lampen, J. O., Bahler, G. P., and Peterson, W. H., *J. Nutrition*, 1942, **23**, 11.

[9] Luckey, T. D., Briggs, G. M., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

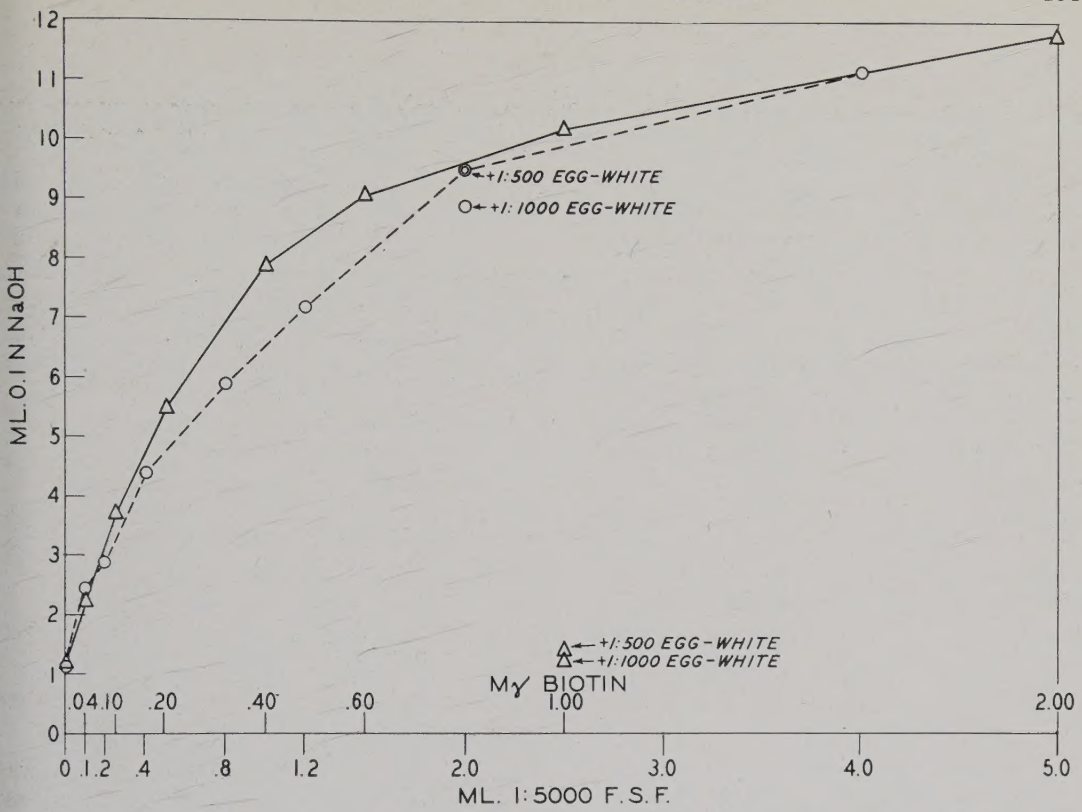

FIG. 1.

The response of *Lactobacillus casei* in an essentially biotin-free medium to the addition of different concentrations of: (1) Biotin (solid line and triangles). Amounts in mγ per 10 ml culture tube given by upper numbers on abscissa, amounts in ml of the standard biotin solution by lower numbers; (2) FSF (broken line and circles). Amounts in ml (ranging from 0.1 to 4.0) of a 1:5000 dilution of a brown oil obtained from hydrolyzed horse plasma. The indicated triangles and circles show, respectively, the growth with 1 mγ biotin plus 2 concentrations of fresh sterile egg-white, and the growth with 2 ml of the 1:5000 FSF plus 2 concentrations of egg-white.

filtered, 98 for the samples similarly treated but brought to pH 9, and 117 for samples which were filtered after being brought to pH 9. The recoveries after autoclaving in 6 N hydrochloric acid or 5 N sodium hydroxide were 79 and 62% respectively.

The biotin-active material which was removed by shaking hydrolyzed plasma with ether could be recovered in the ether extract. This fat-soluble material shall henceforth be designated as FSF. Of the various human plasma protein fractions,* fibrinogen, albumin and γ-globulin contained very little FSF, while the fractions containing the α- and β-globulins and lipids were relatively rich in it. Fractions III-0 and IV-1, 1 W had the highest FSF content, with a biotin activity after acid hydrolysis of about 300 mγ per g. A preparation of FSF from one of these fractions had a quantitatively similar effect on the growth of 3 species of lactic acid bacteria (*Lactobacillus casei, Leuconostoc mesenteroides* and *Streptococcus fecalis* R).

In order to study further the properties of FSF, 10 l of oxalated horse plasma were made 3 N with respect to sulfuric acid and autoclaved for one hour at 15 lbs. The mixture was brought to a pH of about 7.5 with 10 N sodium hydroxide and was filtered

* The plasma fractions were obtained through the kindness of Dr. L. C. Strong of the Harvard Medical School and were prepared under a contract recommended by the Committee on Medical Research between Harvard University and the Office of Scientific Research and Development.

TABLE II.

Effect of Injections of FSF or Biotin in Chicks Fed a Diet High in Egg-White and Inoculated with *Plasmodium lophurae*.

In Experiment 1 the chicks were placed on the special diets at 7 days of age, the twice weekly injections were begun at 10 days and the inoculations were done at 25 days.

In Experiment 2 the chicks were placed on the special diet at 5 days, the injections (twice weekly for biotin, 3 times weekly for FSF) were begun at 8 days, and the inoculations were done at 20 days. In both experiments the injections were intramuscular.

Exp. No.	Diet	Injection	No. chicks	Avg degree of scaliness* of feet and mouth at days 19	25	27	31	35	Avg peak No. of parasites per 10,000 red cells	No. which died within 31 days
1	Egg white 20%	None	3	—	1.8	—	—	2.7	6780	0†
	" " "	Biotin 12 γ per week	4	—	0	—	—	0.1	2165	0
	" " "	FSF-1 γ biotin activity per week	4	—	1.3	—	—	1.6	2505	0
	Casein 20%	None	4	—	0	—	—	0	2730	0
2	Egg white 20%	None	9	0.6	—	2.2	3.8	—	3340	5
	" " "	FSF-1.5 γ biotin activity per week	8	0.2	—	1.0	1.0	—	3175	1
	" " "	Biotin 12 γ per week	8	0.1	—	0	0.2	—	1290	0

* An arbitrary scale ranging from 0 to 6 was used to express the extent of the lesions on the feet and at the corners of the mouth.

† Two of these chicks were very weak at 35 days, when they were killed. All the other chicks in this experiment were active at this time.

through hardened filter paper on a Buchner funnel. The filtrate contained very little activity and was discarded. The black residue was shaken with several large volumes of ether and the yellow ether extracts thus obtained were concentrated *in vacuo*. When all the ether had been removed, 31 ml of a brown oil were obtained. This was liquid at room temperature but solidified to a waxy material when refrigerated. The brown oil (prepared for assay as an opalescent emulsion made by diluting an aliquot with alcohol and then with water or buffer) had a biotin activity of 2.5 γ per ml, and represented a 70% recovery of the total activity of the hydrolyzed plasma. This material was used to demonstrate the ability of *L. casei* to dispense with biotin if adequate amounts of FSF are present. A dilute washed suspension of *L. casei* was inoculated into tubes of biotin assay medium free from added biotin but containing an adequate concentration of FSF. Twenty-four hours later, a loopful of the growth which had occurred was inoculated to 2 similar tubes. Such subinoculation was repeated twice more. The 24-hour growth in the 4th transfer was washed and made into a dilute suspension in the usual manner.[6] This was used to inoculate 2 series of tubes, one containing graded concentrations of biotin, the other graded concentrations of FSF. In each series there were included tubes with adequate biotin or FSF plus more than enough fresh sterile egg-white to inactivate the biotin activity. As is evident from Fig. 1, very similar curves were obtained relating the extent of growth to the relative concentrations of the 2 growth factors. However, the addition of egg-white completely inhibited growth in the tubes with biotin, but had no effect on growth in the presence of FSF. This result indicates that FSF is not merely a stimulatory substance, but can replace biotin in the growth of *L. casei*. All traces of biotin which may have been present in the culture medium must be considered to have been inactivated by the excess of fresh egg-white.

Although the amounts and concentrations of FSF so far available have not permitted a complete test of its effectiveness against egg-white injury in animals, sufficient data have been obtained to show that it prevents the dermatitis produced in chickens by a diet high in egg-white. It also acts like biotin in preventing the increased susceptibility of chickens to infection with the malarial parasite *Plasmodium lophurae*, which otherwise occurs when the animals are maintained on an egg-white diet. The results of 2 experiments are shown in Table II. The diets used consisted of 80% of a chick-starting mash plus 20% of either powdered egg albumin or casein mixed with riboflavin at the rate of 5 mg per 100 g casein. In both experiments, FSF was administered in the breast muscle as the brown oil prepared from hydrolyzed horse plasma. The maximum amount of this which could be injected at one time was 0.2 ml, representing a biotin activity of 0.5 γ. In Exp. 1, the material was injected twice weekly and was reasonably well absorbed between injections. In Exp. 2, where 3 injections per week were given, pockets of the oil formed in the breast muscle. In both experiments the total dosage of FSF, in terms of microbiological biotin activity, was such that an equivalent amount of biotin also would not have given complete protection from biotin deficiency.[10]

All preparations of FSF so far examined have been found to be hemolytic for both duck and sheep red blood cells. The hemolysis is prevented by normal duck plasma. The biotin-like growth activity and the hemolytic activity have gone together through the following preliminary fractionation of the brown oil prepared from hydrolyzed horse plasma. A fraction which was difficultly soluble in alcohol but readily soluble in chloroform had little activity, as did a second fraction soluble in alcohol at room temperature but giving a copious white precipitate from cold alcohol. The active material was soluble in cold alcohol and was non-saponifiable. In the crude state it was soluble in acetone, but after its separation from the inactive saponifiable fraction, it was insoluble in acetone. It is interesting that the addition of 3 parts of acetone to 1 part of an alcoholic solution of the non-saponifiable fraction resulted in a quantitatively equivalent partition of both the growth and the hemolytic activities between the precipitate and the filtrate. Resaponification of the acetone insoluble material again yielded all the activity in the non-saponifiable fraction. The minimum concentration of the various fractions which gave complete hemolysis was equivalent to a biotin activity of 0.2 to 0.3 mγ per ml, when 0.1 ml of 5% red cells was added to 0.9 ml of buffered mixture, incubated ½ hour at 37°C and held overnight in a refrigerator. It is worthy of note that a crude preparation of FSF from a human plasma protein fraction gave complete hemolysis at a concentration with a biotin activity of 0.3 mγ per ml and slight hemolysis at a concentration of 0.15 mγ per ml.

It is apparent from the few properties of FSF thus far known that it cannot be biotin itself, which is not readily extracted in organic solvents[11] and which is inactivated by avidin.[12] FSF also does not correspond to any of the hitherto described analogues or vitamers of biotin, since all of these are either inactive in the growth of *L. casei* and against egg-white injury in animals or, if active, are like biotin itself inactivated by avidin.[13–19]

10 Richardson, L. R., Hogan, A. G., and Miller, O. N., *Univ. Missouri Agric. Exp. Sta. Research Bull.* 343, 1942, 10 pp.

11 Melville, D. B., *Vitamins and Hormones*, 1944, **2**, 29.

12 Eakin, R. E., McKinley, W. A., and Williams, R. J., *Science*, 1940, **92**, 224.

13 Oppel, T. W., *Am. J. Med. Sci.*, 1942, **204**, 856.

14 Burk, D., and Winzler, R. J., *Science*, 1943, **97**, 57.

15 Dittmer, K., and du Vigneaud, V., *Science*, 1944, **100**, 129.

16 Stokes, J. L., and Gunness, M., *J. Biol. Chem.*, 1945, **157**, 121.

17 Pilgrim, F. J., Axelrod, A. E., and Winnick, T., *Science*, 1945, **102**, 35.

18 du Vigneaud, V., Dittmer, K., Hofmann, K., and Melville, D. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 374.

19 Emerson, G. A., *J. Biol. Chem.*, 1945, **157**, 127.

A number of growth-stimulating effects on *L. casei* by naturally occurring fat-soluble substances have been described.[20,21] These effects have been shown to be due to certain fatty acids[22-24] such as oleic acid, which had earlier been identified as a factor essential for the rapid growth of *Corynebacterium diphtheriae*.[25] Oleic acid and related compounds which stimulate the growth of *L. casei* in the presence of suboptimal concentrations of riboflavin or pantothenate do not permit growth in media lacking these vitamins.[22,23] The situation with respect to oleic acid and biotin seems to be rather different.[26,27] *L. casei* is evidently capable of continuous growth in a medium containing only traces of biotin but supplied with an adequate amount of oleic acid and adjusted to an initial pH of 5.6.[27] At first thought one might conclude that the activity of FSF is due to oleic acid, but a close inspection of the available facts makes such a conclusion untenable. Oleic acid in the absence of added biotin produced a maximal growth effect in a medium with an initial pH (before autoclaving) of 5.6. If the pH was 6.5 or higher there was no growth. All the experiments with FSF were routinely done with a medium of pH 6.7-6.8 before autoclaving and 6.2-6.3 after autoclaving. Moreover, in a special experiment, the pH of the assay tubes was adjusted aseptically after autoclaving to 6.8-6.9. Fractions containing FSF had the same biotin activity under these conditions as at a pH of 6.2-6.3. FSF was fully active for *L. casei* even when all traces of biotin in the medium were rendered unavailable by an excess of avidin, a condition which has not been tested with oleic acid. FSF was also active against egg-while injury in chicks, an activity concerning which nothing has been reported for oleic acid. Finally the FSF activity was non-saponifiable and insoluble in acetone, and hence could not have been due to an ordinary fatty acid. It would seem likely, however, that there may be some relation between FSF and oleic acid, and that both are related to the utilization and function of biotin. A knowledge of the chemical nature of FSF may give a new insight into the mode of action of biotin.

Summary. The plasma of various species of animals yields, after hydrolysis with acids or enzymes, a fat-soluble material capable of replacing biotin in the growth of *Lactobacillus casei* and other lactic acid bacteria but not inactivated by avidin. When injected in chickens the material protected them from the injurious effects of a diet high in egg-white. Preparations containing the active material were found to be hemolytic, and in preliminary fractionations the growth and the hemolytic activities have gone together. The properties of the material do not correspond to those of oleic acid or of any previously described vitamers of biotin.

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21 Feeney, R. E., and Strong, F. M., *J. Biol. hem.*, 1942, **142**, 961.

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23 Strong, F. M., and Carpenter, L. E., *Ind. and Eng. Chem., Anal. Ed.*, 1942, **14**, 909.

24 Kodicek, E., and Worden, A. N., *Biochem. J.*, 1945, **39**, 78.

25 Cohen, S., Snyder, J. C., and Mueller, J. H., *J. Bact.*, 1941, **41**, 581.

26 Williams, V. R., and Fieger, E. A., *Ind. and Eng. Chem., Anal. Ed.*, 1945, **17**, 127.

27 Williams, V. R., and Fieger, E. A., *J. Biol. Chem.*, 1946, **166**, 335.

15724

Newcastle Virus: Conversion of Spherical Forms to Filamentous Forms.

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The filamentous forms of Newcastle virus seen in purified preparations[1] have not been seen in the crude allantoic fluid from infected embryos. This despite the fact that the virus is present in the allantoic fluid in the same concentration which in the purified material shows under the electron microscope great numbers of filamentous or tailed forms. Further work here reported indicates that the virus is roughly spherical in the allantoic fluid.

If allantoic fluid from infected chick embryos is centrifuged at 24,000 r.p.m. for 40 minutes and the pellet from this centrifugation is resuspended in water instead of saline, and this procedure repeated for another washing, the virus then shows a great predominance of roughly spherical forms under the electron microscope (Fig. 1b). This water suspension of virus has full activity (Table I) and remains stable for a number of days in the refrigerator (Table II). An occasional virus particle has a long thin attached tail, but gold shadowing fails to reveal other accessory structures. The virus particles are similar to those in the original allantoic fluid (Fig. 1a), with the exception that untreated preparations of infected allantoic fluid have never shown tailed forms.

If to this purified water suspension a little sodium chloride is added, a conversion from the spherical to tailed or filamentous forms occurs (Fig. 2). In the 3 experiments in which these results have been quantitated, the concentration of saline producing conversion has varied from .07 to .15 Molar. The conversion seems to be a progressive process and in individual experiments the extent of this conversion has varied with the salt concentration. Change of form, however, occurs within a few minutes of adding the saline.

Filamentous forms seen in the saline suspensions may be reconverted to the spherical by transferring them back to a water suspension; this is done by centrifuging at 24,000 r.p.m. and resuspending in distilled water. These again will yield filamentous forms on the addition of saline.

The evidence for this conversion or change in form rests entirely on study of dried preparations with the electron microscope, and as yet we have no proof of the existence of these different forms in suspension.

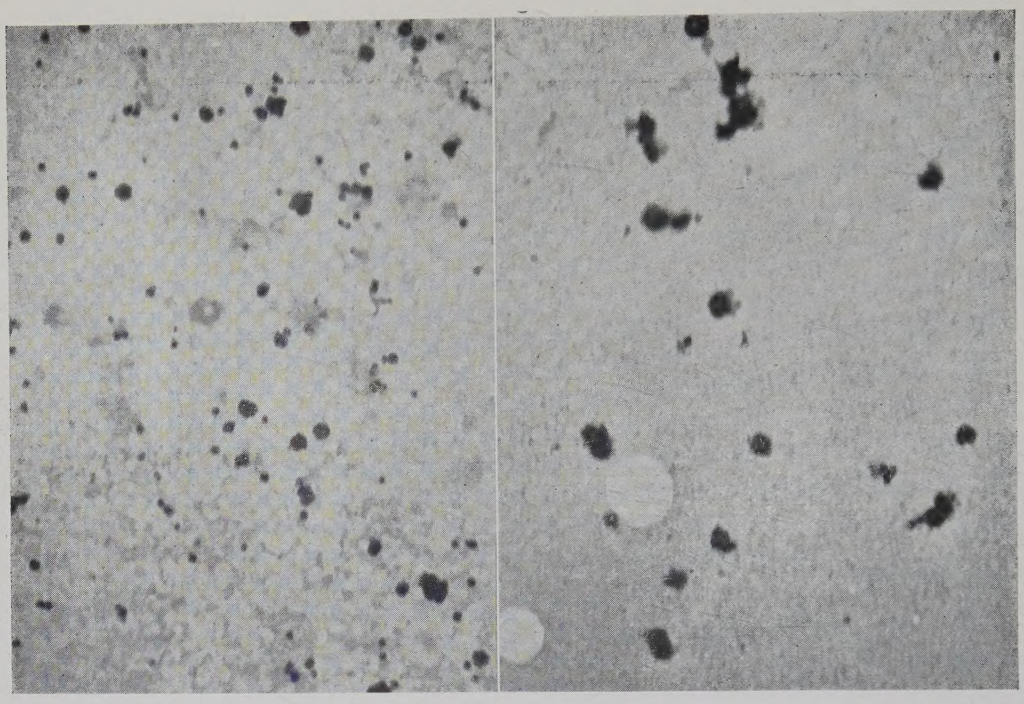
That this conversion is not an artifact, however, is made likely by the following experiments. Dilute solutions (.02%) of formaldehyde progressively inactivate the virus over a period of days. If, an hour or two after formaldehyde has been added, .15 M saline is added, conversion readily takes place. If, however, formaldehyde has been allowed to act for 3 to 5 days before the addition of saline, the stimulus of the saline is no longer able to cause a change in form of most of the particles. Purified preparations in water inactivated by 10^{-3} M or 2×10^{-4} M mustard gas,[2]* or partially inactivated by heating for 10 to 15 minutes at 50°C are also not converted to the filamentous form by the addition of saline. On the other hand, spherical forms which have remained in water without inactivation for as many as 7 or 8 days may be converted to the filamentous form by the addition of saline.

Summary. Study of Newcastle virus in allantoic fluid shows this virus to have a

1 Bang, F. B., Proc. Soc. Exp. Biol. and Med., 1946, **63**, 5.

2 TenBroeck, C., and Herriott, R. M., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 271.

* Dr. R. M. Herriott kindly treated a preparation with these concentrations of mustard gas.



a b

FIG. 1a. Electronmicrograph of unpurified Newcastle virus in allantoic fluid. × 17,200.
FIG. 1b. Electronmicrograph of Newcastle virus resuspended in distilled water after purification by two cycles of differential centrifugation. × 17,200.

TABLE I.
Purification of Newcastle Virus and Resuspension in Water.
Titer: 50% mortality of 10-day embryos.*

Exp. No.	Original allantoic fluid	Virus resuspended in		
		Water	.15 M NaCl	.15 M sucrose
1	$10^{-8.3}$	$10^{-8.7}$	$10^{-8.5}$	
2	$10^{-9.3}$	$10^{-8.8}$		$10^{-9.3}$

* All titrations are calculated on the basis of resuspension in a volume comparable to that of the original allantoic fluid.

TABLE II.
Stability of Newcastle Virus in Water and Saline at 4°C.
Titer: 50% mortality of 10-day embryos.

Time, day	Water	NaCl			Water (2nd exper.)
		.05 M	.15 M	.3 M	
1	$10^{-9.2}$	$10^{-8.6}$	$10^{-9.0}$	$10^{-7.2}$	$10^{-8.7}$
4		$10^{-8.7}$	$10^{-8.2}$	$10^{-6.0}$	
5					$10^{-8.3}$
8	$10^{-8.7}$				

roughly spherical shape when examined under the electron microscope. Most of the particles maintain this form when purified by ultra-centrifugation and by transfer to water. The addition of .07 to .15 M sodium chloride to the solution converts it from the spherical

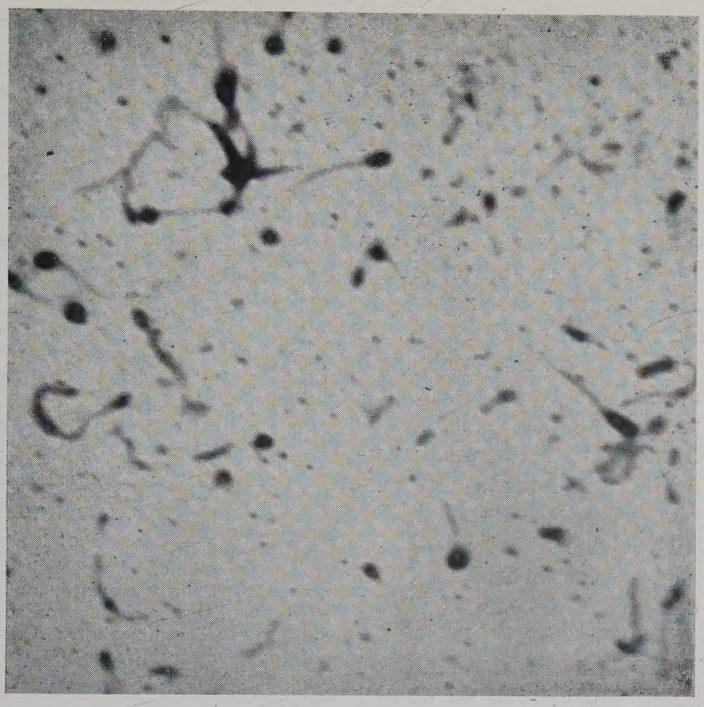


FIG. 2.
Electronmicrograph of same preparation as 1b in .15 M NaCl.
× 17,200.

to the filamentous form previously described. This conversion is prevented by partial inactivation with formaldehyde, mustard gas, or gentle heating.

15725

Effect of Hexaethyl Tetraphosphate on Choline Esterase *in vitro* and *in vivo*.*

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Hexaethyl tetraphosphate, $C_{12}H_{30}P_4O_{13}$, (HTP), has recently been introduced in this country as an insecticide. It was first used in this capacity by the Germans and was uncovered by technical teams following the close of the European phase of the war. The German scientists who were interrogated stated that it had a nicotine-like action and was used for the control of aphids as a substitute for nicotine. The authors of the present report have been unable to find any reference to its mechanism of action other than its nicotinic effects.

The present study was initiated following observation during routine testing[1] that animals showed symptoms similar to those pro-

* Carried out under a contract with Medical Division, Chemical Corps, U. S. Army.

[1] Botkin, A. L., Lipton, M. A., and Mangun, G. H., unpublished data.

[2] Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

duced by diisopropyl fluorophosphate. Muscular twitching, tonic and tonic-clonic convulsions, involuntary defecation, micturition and salivation were observed. The parallelism was further confirmed by the production of miosis by instillation of a dilute solution in the eyes of rabbits. Maximal miosis occurred in about 5 minutes with one drop of solution containing 4 mg/ml of HTP. Pupillary diameter returned to normal in 5-12 hours in contrast to a parallel test with a solution of diisopropyl fluorophosphate (2 mg/ml) in which the miotic effect persisted for 3 days.

Experimental. Cholinesterase was measured manometrically employing a test system containing 0.3 ml 0.1 M acetyl choline bromide in a final volume of 3.0 ml of calcium-free Ringer-bicarbonate buffer (0.025 M $NaHCO_3$, 0.15 M NaCl, and 0.04 M $MgCl_2$). This system contained either 50 mg homogenized brain, 100 mg homogenized submaxillary glands, 100 mg washed diluted red cells, or 100 mg serum. For ascertaining the effect of hexaethyl tetraphosphate (HTP) on insect cholinesterase the entire thorax from cockroaches was employed after removal of the chitin and homogenization. After gassing for 5 minutes with 95% nitrogen-5% carbon dioxide and equilibrating for 10 minutes at 38°C the acetyl choline was tipped from the side-arm into the main compartment of the Warburg vessel and readings were taken at 5-minute intervals for 30 minutes.

The effect of hexaethyl tetraphosphate on the cholinesterase of rat tissues and cockroach tissue *in vitro* was measured by the addition of solutions of the inhibitor dissolved in the buffer to the test system. The inhibitor was added immediately after solutions were prepared and incubated with the tissue throughout the gassing and equilibration period (15 minutes) before the addition of acetyl choline. A final concentration of 1×10^{-7} M hexaethyl tetraphosphate produced the following per cent inhibition of cholinesterase: brain 47, submaxillary 53, serum 60, erythrocytes 45, and cockroach tissue 58. Thus, hexaethyl tetraphosphate was an effective inhibitor of cholinesterase

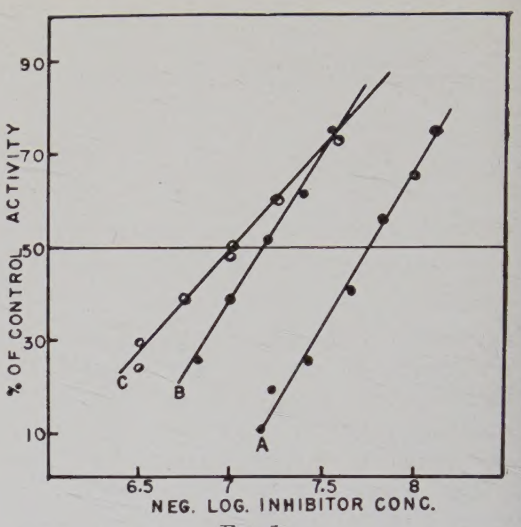

FIG. 1.

The effect of hexaethyl tetraphosphate, diisopropyl fluorophosphate, and a carbamic acid ester on rat brain cholinesterase *in vitro*. Curve A, hexaethyl tetraphosphate; Curve B, di-isopropyl fluorophosphate; Curve C, carbamic acid, N,N-dimethyl-4-dimethylamino-5-isopropyl-phenyl ester, methiodide.

in vitro, all of the tissues showing similar sensitivity toward the compound.

Further evidence of the strong inhibitory action of hexaethyl tetraphosphate on cholinesterase was obtained by comparing its effect on the cholinesterase activity of rat brain *in vitro* with that of diisopropyl fluorophosphate (DFP) and carbamic acid, N,N-dimethyl-4-dimethylamino-5-isopropyl-phenyl ester, methiodide, 2 effective cholinesterase inhibitors. For this comparison a sample of hexaethyl tetraphosphate (Monsanto) and the other compounds were dissolved in the buffer and the measurement carried out as described above. The results shown in Fig. 1 indicate that 50% inhibition of brain cholinesterase activity was obtained by a final concentration of 1.6×10^{-8} M hexaethyl tetraphosphate, 6.3×10^{-8} diisopropyl fluorophosphate, and 1×10^{-7} M carbamic acid ester. Thus, under the conditions of these experiments hexaethyl tetraphosphate was the most effective inhibitor and the similarity in the slopes of the curves in Fig. 1 indicate a similar type of inhibition by DFP and HTP.

Inhibition of cholinesterase *in vivo* was

TABLE I.
Effect of Hexaethyl Tetraphosphate on Cholinesterase Activity of Rat Tissues *in Vivo*.

Dose, mg/kg	% inhibition of activity			Toxicity
	Brain	Submaxillary	Serum	
1	4.5	22	100	0/3 died in 20 minutes
2	45.8	56.5	100	1/3 " " 20 "
3	75.8	83	100	2/3 " " 20 "
5	100	100	100	3/3 " " 5-6 "
10	100	100	100	" " 5 "

demonstrated by administering HTP intraperitoneally to rats and then measuring the cholinesterase activity of the brain, submaxillary glands, and serum by the *in vitro* test system previously described.

The results of these measurements are given in Table I. Values at 1, 2, 3, and 5 mg per kg are the averages of determinations on 3 animals and the 10 mg per kg represents one animal. The cholinesterase values for normal tissues from 10 animals expressed as microliters CO_2 per 50 mg fresh tissue per 10 minutes were: brain 102, submaxillary glands 28, serum 10, and red blood cells 7 and the per cent inhibition in poisoned animals was calculated from these control values.

These *in vivo* experiments demonstrate that HTP inhibits the cholinesterase of all of the tissues examined. Most sensitive was the serum which was completely inhibited by doses of the compound which produce muscular twitching but no lethal effects in the animals. The inhibition of brain and submaxillary gland cholinesterase more nearly paralleled the symptoms and lethal action of the drug with submaxillary gland esterase being inhibited to a somewhat greater extent than brain. Twenty-four hours after the injection of a sublethal dose (1 mg per kg) into rats 35% of the serum activity had returned, at 4 days 70% of the serum activity had returned, and at 8 days the activity had returned to normal.

Summary. Hexaethyl tetraphosphate exerts a strong inhibitory effect on mammalian and insect cholinesterase *in vitro* and *in vivo*. This finding, in conjunction with its gross effects on animals suggests that its physiological effects may be at least in part due to its inhibition of this enzyme.

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Antigenic Structure of *Pasteurella pestis* and the Isolation of a Crystalline Antigen.*

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The present report is a summary of investigations initiated in 1942 to extend the knowledge of the antigenic structure of *P. pestis*, and to use this information to place plague prophylaxis on a sounder basis. The detailed experimental data will be reported at a later date.

All the studies, unless otherwise stated, were performed with dried plague bacilli of the virulent "Yreka" strain. The bacilli were grown on agar for 3 days at 37°C and suspended in saline. This was precipitated by one to 2 volumes of acetone cooled to

* The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of California.

—70°C and left overnight. Repeated washings with acetone and drying *in vacuo* produced a bacterial powder of high antigenicity and toxicity (LD_{50} for 20 g mice: 20-40 μg).

Extraction of the acetone-dried plague bacilli with neutral salt solutions (0.85%, 2.5% sodium chloride solutions or 0.4% sodium acetate solution) yielded a water-soluble and a water-insoluble antigenic component. The water-soluble fraction is quite toxic for mice and rats (LD_{50} for 20 g mice, 8-15 μg), and has a high immunogenic value for these species, but a low value for guinea pigs. The water-insoluble portion is nontoxic for mice, and produces a very poor immunogenic response in mice and rats. However, this fraction, when tested as an alum precipitate, has a high immunogenic value, equal to that of whole plague bacilli, for guinea pigs.

The *water-soluble fraction* has been shown to contain at least 3 antigenic components: (1) A carbohydrate protein-soluble at 0.25 saturation of ammonium sulfate at pH 7.0-7.5, and precipitable at 0.3 saturation (Fraction IA). (2) A carbohydrate-free protein soluble at 0.3 saturation of ammonium sulfate at pH 7.0-7.5, and which crystallizes in the form of fine needles when the concentration of ammonium sulfate is raised to 0.33 saturation (Fraction IB). (3) A toxic fraction soluble at 0.33 saturation of ammonium sulfate at pH 7.0-7.5 and almost completely precipitated at 0.55-0.67 saturation (Fraction II). Both Fractions IA and IB are electrophoretically homogenous at pH 8.7 and pH 5.5. They both have approximately the same mobility at both pH values. Fraction IA has the following analytical values: Nitrogen 15.07%, sulfur 0.58%, phosphorus 0.08%, a strongly positive Molisch test, and is highly viscous. Fraction IB has the following analytical values: Nitrogen 15.71%, sulfur 0.59%, phosphorus 0.04%, a negative Molisch test, and a low viscosity. Both fractions are free of agar as determined by serological tests with a sample of horse serum containing antibodies to agar.

Fractions IA and IB are similar immunogenically. They both produce potent antisera in rabbits, and will absorb all of the antibody from sera prepared against either. Sera to these fractions agglutinate plague bacilli to at least the same titer as sera prepared against whole plague bacilli. In one experiment, a serum to Fraction IA had as high a protective value for mice as serum to whole bacilli but, in contrast, had a very low protective titer for rats when compared with serum to whole bacilli. Serum to either of these fractions is incapable of neutralizing plague toxin. Both fractions will induce immunity in mice. Neither fraction is of value in producing immunity in guinea pigs.

Fraction II has never been obtained in a state approaching chemical or immunological purity. All preparations have been contaminated with Fraction I (IA and IB) in sufficient quantity to produce antibodies to Fraction I in rabbits. The best preparations had a toxicity (LD_{50}) for 20 g mice of 0.6 μg. However, it has been possible to prepare toxins free of Fraction I by serological technics. It was observed that plague bacilli grown at room temperature were fully toxic, but extracts of such bacilli contained very little Fraction I. It proved possible to remove the residual Fraction I by absorption with either Fraction IA or IB antisera. The resulting absorbed extract showed no decrease in toxicity, and produced antisera in rabbits capable of neutralizing plague toxin. However, this antiserum was devoid of protective value for mice, did not agglutinate antigenically complete plague bacilli to a significant titer, nor react with either Fraction IA or IB except to a very slight degree in sensitive ring tests. Its value in inducing immunity in mice and guinea pigs has not as yet been investigated.

From the *water-insoluble fraction* or "residue" only small amounts of protein can be extracted with mild alkalies. Anhydrous phenol liquefied with 10-15% acetone dissolves approximately 25% of the residue. Both fractions, the phenol-soluble as well as the insoluble one, contained the antigen which protects guinea pigs.

The 2 soluble atoxic antigens (Fractions IA and IB) undoubtedly represent the "en-

velope" antigen of Schütze[1] and others. They are probably present on the surface of the bacilli, because antisera prepared against these fractions will agglutinate the strains of plague bacilli thus far tested. Only one avirulent strain, TRU,[1] obtained from Dr. Harry Schütze, Lister Institute, London, England, is not agglutinated by Fraction I sera, although it is slightly agglutinated by sera prepared against whole plague bacilli. This strain appears to be devoid of these 2 fractions. The exact relationship between Fractions IA and IB is not clear. Serologically, they are almost identical. Thus far, the crystalline Fraction IB proved of slightly lower immunogenic value. It might be suggested that in the intact cell the normal antigen is the carbohydrate protein Fraction IA, and that the crystalline protein is an artifact formed by disaggregation of the molecule during the death of the cell and subsequent treatment. All attempts to alter Fraction IA short of denaturation have failed. It may be that plague bacilli contain an enzyme capable of converting Fraction IA to IB. Serologic studies have shown that Fraction I (IA and IB) is formed by the virulent strains tested, and the avirulent strain 1122 which forms a stable suspension in saline; avirulent strains showing salt instability produce only traces. These antigens are formed in quantity at 37°C; extracts of cells grown at room temperature contain relatively small amounts.

In view of the observed differences in response of the various laboratory animals to the different antigens of plague bacilli, it is considered advisable at the present time to use vaccines containing all of the antigens of *P. pestis* for prophylaxis of plague in man, and for the production of antiplague serum for therapeutic use.

The authors wish to thank Dr. M. Heidelberger, College of Physicians and Surgeons, for the gift of a sample of anti-influenza horse serum containing antibodies to agar; Dr. A. Elik, The Rockefeller Institute for Medical Research, for the microanalysis; and Dr. T. Shedlovsky, The Rockefeller Institute, for the electrophoretic analyses.

1 Schütze, H., *Brit. J. Exp. Path.*, 1932, **13**, 284; 1939, **20**, 235.

15727

Liberation of Histamine and Heparin by Peptone from the Isolated Dog's Liver.*

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In previous reports[1–3] one of us has shown that several agents (antigen, *e.g.* horse serum in the sensitized animal, or peptone or Ascaris extracts) which *in vivo* produce a shock-like syndrome in the dog associated with a discharge of histamine and heparin from the liver, are unable to liberate significant amounts of these substances when perfused through the isolated liver, using Tyrode's solution as a vehicle. When citrated blood or heparinized blood is used as the perfusion fluid, detectable quantities of histamine and sometimes of heparin are discharged, but the total amounts that can be found in the perfusates are far smaller than those which are discharged in experiments *in vivo*. These

* Aided by a grant from the John and Mary R. Markle Foundation.

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1 Rocha e Silva, M., and Grana, A., *Arch. Surg.*, 1946, **52**, 713.

2 Rocha e Silva, M., Porto, A., and Andrade, S. O., *Arch. Surg.*, 1946, **53**, 199.

3 Rocha e Silva, M., and Teixeira, R. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 376.

results lead to the conclusion that whole blood is necessary to produce the discharge of histamine and heparin from the hepatic cells in the forms of shock mentioned. In the experiments to be reported here, it has been possible on occasion to obtain the liberation from the liver of amounts of histamine and heparin greater even than *in vivo* and hence it has been possible to suggest the nature of the factors necessary for the liberation of these substances from the liver.

Methods. Ten dogs were used in the present experiments, all of them anaesthetized with sodium pentobarbital ("Ibital," Ingram and Bell). The liver was prepared for perfusion as described previously.[2] To avoid the use of anticoagulants in the blood, glassware treated with the silicone (Dri-Film, No. 9987, General Electric Co.) was used. In vessels so coated, blood will remain unclotted for 2 or 3 hours and the platelets are preserved for at least 30 minutes.[4] It was assumed that, by thus completely excluding anticoagulants, conditions would be obtained more closely resembling those *in vivo*. All glassware entering into contact with the blood was carefully coated with the silicone, as described elsewhere,[4] and contact with rubber tubing was reduced to a minimum. The blood was collected from the carotid through cannulae treated with silicone. Care was exercised to minimize trauma to the artery. Only the first 400 ml of blood were used for the perfusion. As soon as the animal had been exsanguinated, a cannula was inserted into the portal vein and the liver was perfused with warm (38°C) Tyrode's solution. The entire left lobe of the liver was tied off and was excised. The remaining portion of the liver was isolated and was placed in a warm chamber. The perfusion with warm Tyrode's solution was continued for 5 to 10 minutes, until the perfusate was free of blood. Three g of peptone in 10 ml of saline were then added to 250 ml of blood and the mixture was pumped rapidly through the organ. The perfusate was collected in small portions of 20 ml each and was assayed for histamine and heparin. The organ was perfused further with 200 ml of blood and finally was washed out by prolonged perfusion with Tyrode's solution.

For the estimation of the histamine content of the perfusates, a piece of guinea pig ileum was employed. The heparin content was determined by the protamine-titration method of Jaques and Waters.[5] In the experiments in which Tyrode's solution was used as the vehicle, the presence of heparin was investigated by concentrating the perfusates and then testing for metachromasia with the dye, Azure A.[6] By this method, heparin can be estimated quantitatively in concentrations as low as 0.02 mg/ml, while it can be detected qualitatively with concentrations as low as 0.0005 mg/ml. We are indebted to Mr. E. Napke for conducting this test. The figures for total histamine and heparin given in Table I cover the whole discharge, since the perfusates were collected until only traces of histamine were detected by the biological test. In some of the experiments, after the perfusion with blood, a piece of the liver was taken in a 3.8% solution of sodium citrate and smears were prepared according to a technic previously described,[2] to observe the degree of disintegration of the clumps of platelets found within the liver parenchyma after perfusion with peptone. The same technic applied to the normal liver shows only intact, isolated platelets. Red cells, leucocytes and platelets were counted in the perfusing blood in 6 of the experiments and the degree of agglutination is indicated as the percentage of aggregated platelets to the total count.

The peptone was a sample of proteose-peptone (Difco) in 30% solution. n previous experiments we used a peptone which contained histamine as an impurity and it was necessary to rely upon increases in the histamine content of the perfusing blood. In these experiments the histamine was removed from the peptone by treatment with permutit,

[4] Jaques, L. B., Fidlar, E., Feldsted, E. T., and Macdonald, A. G., *Can. Med. Assn. J.*, 1946, **55**, 26.

[5] Jaques, L. B., and Waters, E. T., *J. Physiol.*, 1941, **99**, 454.

[6] Jaques, L. B., Mitford, M. B., and Macdonald, A. G., to be published.

TABLE I.
Total Histamine and Heparin Released from Isolated Dog's Liver Following Addition of Peptone to the Perfusion Fluid.

Dog, No.	Wt, kg	Liver, g	Perfusion fluid	Histamine, μg	Heparin,† mg
11	16.0	170	Tyrode's solution	26.8	None
17	15.5	402	" "	38.0	Traces
"	"	"	Heparinized blood—45 min. contact (8 units/ml)	93.7	1.75
19	17.6	365	Defibrinated blood	1,162.0	Not est'd
18	12.5	365	Heparinized blood—22 min. contact (8 units/ml)	538.0	Doubtful
16	10.1	295	" " 15 " " 5 "	2,511.0	10.5
13	22.5	280	Silicone blood‡	89.0	3.65
15	8.3	140	" "	417.7	5.45
14	29.6	359	" "	607.7	8.15
12	16.2	—	" "	3,313.0	16.5
20*	23.0	470	" "	8,550.0	25.4

* The figures for histamine and heparin in Dog 20 would not cover the whole discharge since after stoppage of the drainage with Tyrode the histamine was still high (2.8 μg per ml) and the heparin was estimated only in the tubes containing enough blood to be used in the protamine assay (350 ml of perfusate).

† The ratio of heparin : protamine under the conditions of this experiment was found to be 1:2.

‡ Blood collected and preserved in silicone-coated vessels.

as described by Gotzl and Dragstedt,[7] thus permitting a much more accurate estimation of the histamine eventually liberated in the perfusion experiments. When the solution was diluted 25 times, as in the perfusion experiments, and was tested upon the guinea pig gut, only traces of histamine were found. It should be noted that a larger quantity of peptone was used in the present experiments than in those referred to above. Although we formerly used 1 or 2 g of peptone for 500 or 600 ml of blood (diluted 20% with Tyrode's solution), in the experiments presented in this paper we have used 3 g of peptone added to 250 ml of undiluted blood. This difference was partly due to the fact that the sample of peptone used appeared to be less active than previous samples. The trypsin used was a crystalline sample containing 50% of $MgSO_4$ and produced by the Lehn and Fink Corporation. The protamine was a sample of salmine hydrochloride supplied by the Connaught Laboratories, University of Toronto.

Results. The total quantity of histamine and heparin liberated from the isolated liver of the dog, following the addition of peptone to the perfusion fluid, is presented in Table I. In agreement with results reported previously, peptone is capable of liberating only small amounts of histamine and traces of heparin when Tyrode's solution is used as the perfusing fluid. It is possible that even this small amount of histamine is liberated because of the presence of traces of blood retained within the perfused organ, since after the injection of peptone some blood appeared in the perfusate. In one experiment, the effect of crystalline trypsin on the perfused liver was tested. After washing the liver of Dog 11 free from peptone and traces of histamine with Tyrode's solution, 50 mg of crystalline trypsin was added to the Tyrode's solution perfusing the liver; 132 μg of histamine and 1.5 mg of heparin were then released by the liver.

In contrast to the results obtained on adding peptone to Tyrode's solution, when whole blood (especially blood kept unclotted by the use of silicone instead of anticoagulants) was used as a vehicle, enormous amounts of heparin and histamine were liberated. As shown in Fig. 1, most of the histamine and heparin was released at the beginning of the perfusion with blood and peptone. In some of the experiments the amount of histamine in the perfusates went up as high as 40 or 50 μg per ml, in contrast to the 1.5 μg per ml that has so far been the maximal content of histamine estimated in the blood of intact animals submitted to peptone shock.[3] The variability shown in Table I in

[7] Gotzl, F. R., and Dragstedt, C. A., *J. Pharm. and Exp. Ther.*, 1941, **74**, 33.

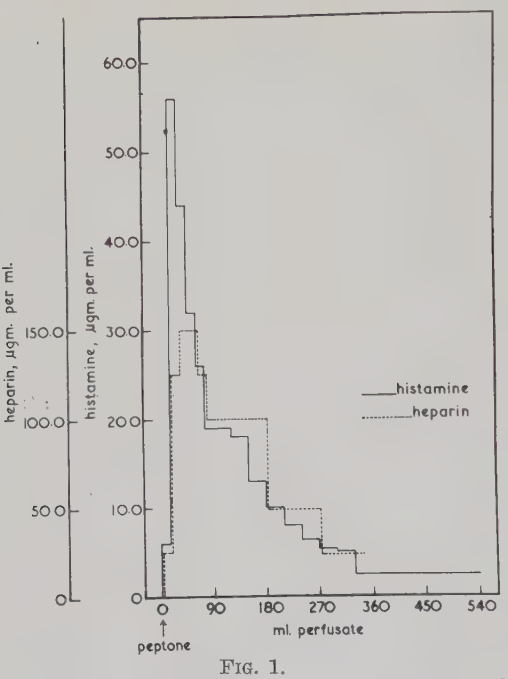

FIG. 1.

Dog No. 20. Liver perfusion with silicone blood + peptone. Discharge of histamine and heparin after passage of the blood through the liver. Note the sharp peak (56 μg histamine per ml of perfusate) occurring before the first 50 ml of blood passed through.

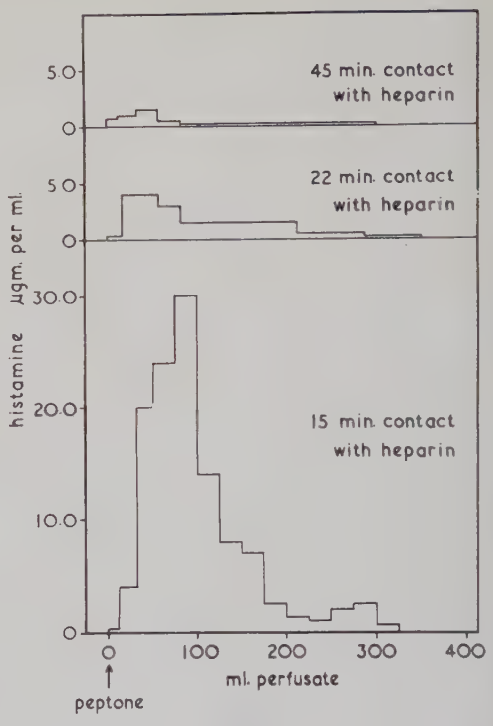

FIG. 2.

Three experiments with heparinized blood peptone. The maximal discharge of histamine was obtained when the blood was used 15 minutes after addition of heparin and the minimal when the blood was maintained for 45 minutes in contact with the heparin before passing through the liver.

the amounts of histamine and heparin released when "silicone blood" was used in the perfusion suggests that other factors (individual differences in the response of the liver from different dogs, the time of contact of the blood with peptone before coming in contact with the cells of the liver, etc.) are also operative in these experiments. These factors will be the subject of a separate investigation.

In the experiments presented in Table I even heparinized blood with peptone was able to liberate amounts of histamine and heparin far more conspicuous than those detected in previous experiments.[3] This might be due to one or more of the following reasons: (1) the peptone was freed from histamine by treatment with permutit, thus permitting a much more accurate assay of histamine; (2) much higher doses of peptone were used than before; (3) the blood used was undiluted, while in the previous experiments the perfusion solution was blood which contained approximately 20 to 25% Tyrode's solution. Whatever may be the reason for this discrepancy, heparin appeared to afford some protection against the action of peptone when enough time was allowed to elapse between the addition of heparin and the start of the perfusion experiment. As shown in Fig. 2, the maximal discharge of histamine was obtained when the blood remained in contact with heparin only 15 minutes. The minimal discharge resulted when the blood was kept for 45 minutes in contact with heparin. Due to the small number of experiments with heparin, further work is required to establish that heparin inhibits the discharge of histamine from dog's liver. Although defibrinated blood also permits the release of appreciable amounts of histamine and probably also of heparin (not estimated) by peptone, there is no question that the use of silicone-coated vessels for the collec-

TABLE II.
Leucocyte and Platelet Counts per mm³ of Perfusing Blood.*

Dog No.	Normal in silicone		After heparin		After peptone		Perfusate	
	Leucocytes × 100	Platelets × 1000	Leucocytes × 100	Platelets × 1000	Leucocytes × 100	Platelets × 1000	Leucocytes × 100	Platelets × 1000
15	62	411 (6%)†	Heparin not used		135	398	49	50 (87%)
16	77	415	79	368 (16%)	48	151 (67%)	41	70 (79%)
17	45	341	—	—	59	187 (91%)	5	12 (15%)
18	102	291	82	140 (42%)	94	227 (44%)	10	32 (25%)
19	25	363	79	46 (11%)	35	24 (62%)	14	14 (54%)
20	77	171	Heparin not used		74	129 (64%)	32	8.7 (48%)

* The red cells were used as an indicator of accuracy of sampling and of dilution, and in each experiment the leucocyte and platelet counts have been adjusted to the count of red cells in the normal blood.
† The figures in brackets indicate the degree of agglutination.

tion of blood, which thereby may approach its natural condition, permits the discharge of histamine and heparin in far more significant amounts than those obtained with defibrinated or heparinized blood.

Since the liberation of histamine and heparin from the liver by peptone appears to depend on the presence of whole blood, the question arises as to what constituents of the blood are involved in this action. It was previously found[2] with citrated blood as the perfusion fluid and antigen (horse serum) or Ascaris extracts as exciting agents that a sharp decrease in platelet and leucocyte counts occurred on passing the blood through the liver. As shown in Table II (Dogs 15, 17 and 20), this likewise occurred with the blood in silicone. Since the addition of peptone to the blood caused a marked agglutination of the platelets, the decrease in count may be attributed to trapping of these clumps in the hepatic capillaries. Confirming previous reports by Copley,[8,9] heparin itself, when added to blood, caused considerable clumping of platelets and sometimes a sharp decrease in the count. The presence of heparin had little apparent effect on the action of peptone on the platelets and on the further decrease in the count when the blood passed through the liver.

Counts of leucocytes and of platelets cannot afford any direct evidence concerning the disintegration of these cells, since they might be segregated in the network of capillaries and small vessels of the liver after forming clumps on the addition of peptone, as shown in Table II. Information regarding the destruction of these clumps was afforded by the observation of smears taken from pieces of the organ after the perfusion. In the experiments in which an excess of heparin had been used, the platelet clumps could be seen in great numbers throughout the slides, while in those experiments in which there was a considerable discharge of histamine and heparin the clumps were either absent or heavily damaged. The remains of platelets were seen but only very seldom were there any well stained clumps. This suggests that a disintegration of the platelets is associated with the liberation of histamine and heparin, and that while heparin will clump platelets, it will prevent their later disintegration.

The present report is intended to describe conditions under which it is possible to obtain regularly the liberation of histamine and heparin from the dog's liver with peptone. Using this technic we hope to elucidate the mechanism for this liberation. Rocha e Silva and Teixeira have postulated the activation of plasma trypsin as an intermediary in the liberation of histamine and heparin. The finding that the mechanism with peptone depends on the presence of blood, whereas crystalline trypsin by itself was observed to cause some release of histamine and heparin, is suggestive in this regard.

Conclusion. Experiments on the perfusion of dog's liver show that the presence of blood

8 Copley, A. L., *Am. J. Physiol.*, 1942, **133**, 248.

9 Copley, A. L., and Robb, T. P., *Am. J. Clin. Path.*, 1942, **12**, 416.

in the perfusing fluid is important for the production of an appreciable discharge of histamine and heparin by peptone. The best results were obtained when anticoagulants were excluded and the blood was preserved from clotting in silicone-treated receptacles. When Tyrode's solution was used as a vehicle for the perfusion, only small amounts of histamine and traces of heparin appeared in the perfusates. The experiments presented in this paper do not afford any direct proof but are suggestive of the participation of platelets and possibly also of leucocytes in the mechanism of the discharge of histamine and heparin from the liver.

The authors are greatly indebted to Professor C. H. Best for facilities provided in the Department of Physiology, University of Toronto, and for his interest and support.

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Effect of Reticulo-Endothelial Blockade on Immunity to the Shwartzman Phenomenon.*

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Experiments in this laboratory have shown that repeated intravenous injections of typhoid vaccine cause rabbits to develop a tolerance to the pyrogenic effect of the vaccine, and, furthermore, that this tolerance can be abolished by reticulo-endothelial (R-E) blockade.[1] Additional studies have shown that the development of tolerance to typhoid vaccine carries with it a similar tolerance to the pyrogenic effects of certain other Gram-negative bacilli, not serologically related to the typhoid bacillus.

In view of the fact that the pyrogenic activity of several species of Gram-negative bacilli has been shown to be the property of a carbohydrate component[2,3] and that, at least in the case of *B. prodigiosus,* the same carbohydrate has been shown to be capable of inducing the Shwartzman phenomenon,[4] it was thought that a study of immunity to that form of injury might throw some light on the general problem of immunity to pyrogens. As a tool for study of this problem the Shwartzman phenomenon is advantageous because it provides an observable tissue injury in an area containing few R-E elements.

Materials and Methods. The source of bacterial toxin was the "Mitchell" strain of *E. typhosa.*† An "agar washings" filtrate was prepared by Shwartzman's method.[5a] The potency of the filtrate was determined by titrating the minimum intravenous reacting dose against a constant skin preparatory dose.[5b] Two different lots were prepared. Titration of the first showed 1 reactive unit in 1 ml of a 1:300 dilution, per kg of body weight; while in the second, 1 reactive unit was contained in 1 ml of a 1:400 dilution, per kg of body weight. The skin preparatory dose used in titrations and in all experiments was 0.25 ml of undiluted filtrate.

The rabbits were males, weighing 2 to 3

* Aided by a grant from the United States Public Health Service.

1 Beeson, P. B., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 248.

2 Robinson, C. S., and Flusser, B. A., *J. Biol. Chem.*, 1944, **153**, 529.

3 Hartwell, J. L., Shear, M. J., and Adams, J. R., Jr., *J. Nat. Cancer Inst.*, 1943, **4**, 107.

4 Shwartzman, G., *Cancer Res.*, 1944, **4**, 191.

† Obtained from the Laboratories of the Georgia Department of Public Health.

5 Shwartzman, G., *Phenomenon of Local Tissue Reactivity and Its Immunological, Pathological, and Clinical Significance,* New York, Hoeber, 1937, (a) p. 35, (b) p. 26.

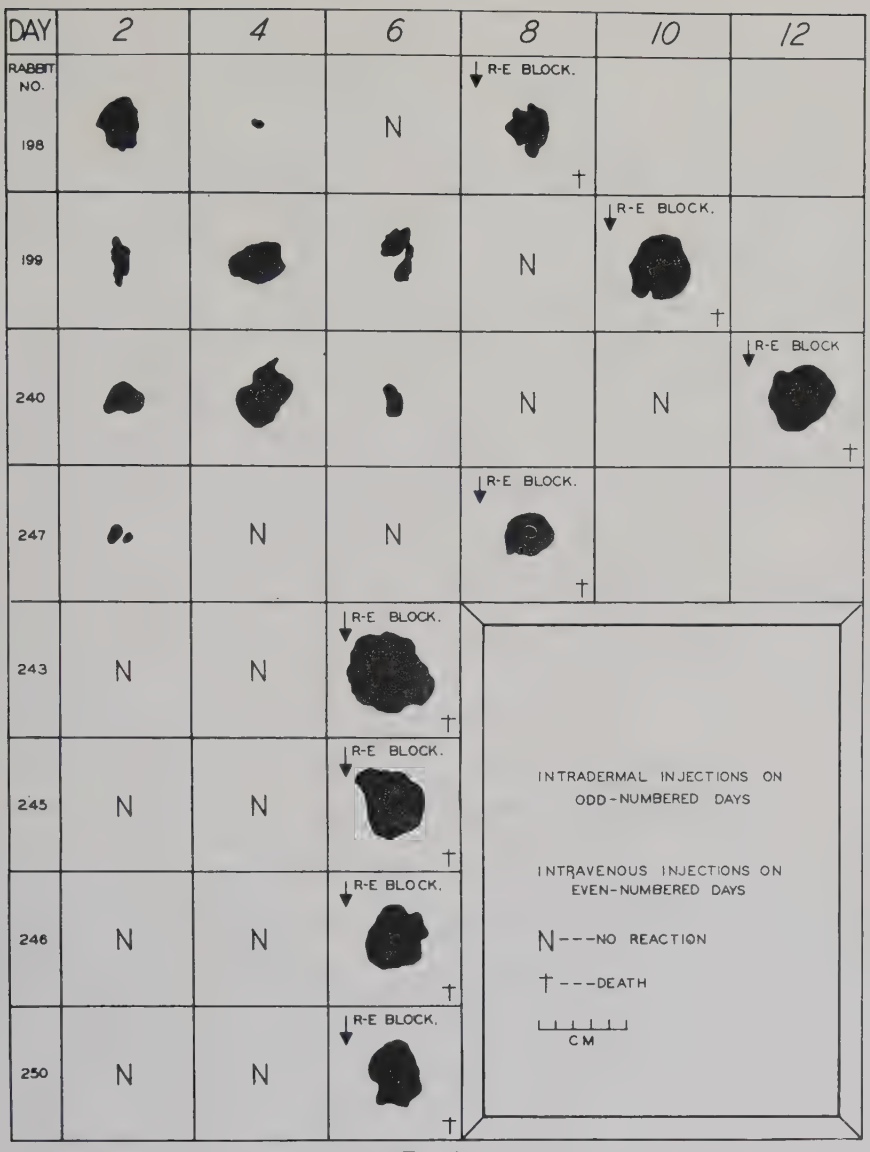

FIG. 1.

Graphic representation of the results obtained in 8 of the 20 rabbits given Thorotrast blockade. The upper 4 rabbits received the blockade after acquiring immunity, while the lower 4 were naturally immune. The shaded areas represent the area of hemorrhagic necrosis 5 hours after the intravenous injection of toxin. The scale in centimeters is indicated.

kg, either Chinchillas or New Zealand whites (Rockland). Tests were made on the skin of the abdomen. Since each animal was to have a series of tests, the abdomen was marked to designate 8 areas, and in the group of animals used in each experiment the individual tests were placed at different locations. The purpose of this was to lessen the possibility of error due to injection in areas of low reactivity, since Shwartzman has found that there are differences in skin reactivity in different parts of the abdomen. Subsequent injections in each animal were made in adjacent areas, moving in a clockwise direction. The intravenous, or provocative dose, was 20 reacting units; this was admin-

istered 22 hours after the intradermal injection. The result was read 5 hours later. The only criterion used in determining the reaction was the presence or absence of a purplish hemorrhagic lesion. When positive reactions occurred the areas of skin hemorrhage were traced on transparent X-ray film, and then transcribed to permanent records.

For R-E blockade 2 agents were used: Thorotrast and trypan blue. Thorotrast is a commercial preparation (Heyden Co.) containing approximately 25% colloidal thorium dioxide. This was administered intravenously, in a dosage of 9 ml, 6 hours after the preparatory intradermal inoculation. Trypan blue was given in a 1% aqueous solution, the dose being 6 ml. This was given twice, 6 and 20 hours after the preparatory intradermal inoculation.

The plan followed in the experiments was to elicit the Shwartzman phenomenon in an animal repeatedly, the intradermal injection of one test being given on the day following the intravenous injection of the previous test. When an animal showed evidence of immunity by failing to react, the procedure was repeated with the addition of R-E blockade. In most of the experiments 2 negative results were obtained before the blockade, but in a few instances this was done after only one negative test.

Results. Of the 28 rabbits used in these experiments 13 were naturally immune; that is, no hemorrhagic reaction was visible 5 hours after the intravenous injection of 20 reacting units. The remaining 15 animals reacted positively 1 to 4 times before they too became immune.

Ten of the rabbits that were naturally immune received Thorotrast as a blocking agent, after 1 or 2 previous negative tests. In every case a positive reaction resulted. Ten rabbits that initially reacted positively but then showed negative reactions after 1 to 3 repetitions of the procedure also received R-E blockade with Thorotrast. Again, in every instance a positive reaction resulted. Fig. 1 shows a diagram of the results obtained with Thorotrast blockade in 8 of these animals, 4 naturally immune, and 4 with acquired immunity. Not only did R-E blockade cause the development of large areas of hemorrhagic necrosis at the sites of the intradermal inoculations, but also it caused death of all of these 8 animals.

When trypan blue was used as the blocking agent the results were not as uniform. In 3 naturally immune animals, blockade with this agent resulted in only one positive reaction. In 5 rabbits which had acquired immunity, blockade caused positive reactions twice.

The dose of bacterial toxin used in these experiments did not cause a single death in approximately 75 tests in this group of animals. Yet when R-E blockade was combined with the same dose of toxin, 17 of the 20 animals that received Thorotrast, and 3 of the 8 rabbits given trypan blue, died within 24 hours after injection of the intravenous dose of toxin. It is obvious then that R-E blockade not only alters the course of events at the site of the preparatory skin injection, but also that it markedly impairs the animal's general defense against the bacterial toxin.

The 3 animals which survived after exhibiting the Shwartzman phenomenon with Thorotrast blockade were retested immediately without blockade, and all 3 reacted negatively. Two of these, on receiving a second test combined with Thorotrast, again reacted positively and died within the succeeding 24 hours. The third survived a long sequence of tests, wherein it received a blocking dose of Thorotrast on 5 separate occasions. Following the last blockade it continued to react positively to a series of 10 tests, apparently being unable to regain a state of immunity. The reason for the unusual resistance of this animal, and for its eventual inability to become immune, is not clear.

Discussion. The results obtained with Thorotrast blockade in these experiments, and in our previously reported work on the febrile response to bacterial toxins[1] have been clear-cut and readily reproducible. This can be attributed partly to the fact that in both types of experiment the phenomenon being studied takes place within a few hours, allowing a test to be completed during a time

when functional impairment of the R-E system is at its height. Prolonged R-E blockade is almost impossible, because of the rapid recovery of function by the phagocytic cells and because of the fact that blockade is in itself a powerful stimulus to proliferation of these cells.[6]

Consideration of these results leads to the assumption that immunity to the Shwartzman reaction depends on ability of the R-E system to remove the bacterial toxin from the blood stream to such an extent that the tissues at the site of skin preparation are spared serious injury. R-E blockade permits the toxin to be delivered to the prepared skin area in a concentration sufficient to produce capillary damage and hemorrhagic necrosis. It also appears that other tissues suffer more extensive damage from the toxin in the presence of R-E blockade, as evidenced by the high fatality rate associated with blockade. Neither Thorotrast nor trypan blue would cause death in the doses used here.

These findings indicate that the functional state of the R-E system is of great importance in either natural or acquired immunity to the Shwartzman phenomenon. Shwartzman and others have provided a considerable body of evidence which indicates that specific antibodies may play an important part in acquired immunity. In view of the fact that phagocytosis by R-E cells is known to be enhanced in the presence of specific antibody, the 2 types of evidence do not necessarily conflict. Nevertheless, the evidence obtained in our other studies on immunity to bacterial pyrogens indicates that an acquired immunity can develop without the participation of specific humoral antibodies.

It is of interest that Thorotrast was much more effective in this action than was trypan blue. The dosages of both agents were comparable to those which have been used by other workers in studies of R-E blockade. One should note, however, that the actual quantity of colloidal thorium dioxide given was approximately 20 times that of trypan blue.

Previous work on the effect of R-E blockade on the Shwartzman phenomenon has been somewhat contradictory. Gratia and Linz reported that blockade with large doses of India ink neither intensified nor lessened the local skin reaction.[7] Two Italian reports, on the other hand, state that R-E blockade inhibits the Shwartzman phenomenon.[8,9] In one of these studies trypan blue was used as the blocking agent, while in the other lithiocarmine was used. The findings reported here are in direct opposition to the conclusion that R-E blockade inhibits the Shwartzman phenomenon.

This work was undertaken principally in an attempt to elucidate the nature of the immunity developed by human beings and animals to the fever-producing effects of certain bacterial toxins. It would appear, however, that the findings may be of some use in other work involving the Shwartzman phenomenon, in that the variations in reactivity of different test animals can probably be lessened by the use of R-E blockade.

Summary. A study has been made of the effect of R-E blockade upon natural or induced immunity of rabbits to the Shwartzman phenomenon. Striking results were obtained when Thorotrast was used as the blocking agent. Rabbits that had shown a natural immunity, or had become immune following a series of previous Shwartzman reactions, responded, after the injection of Thorotrast, by developing typical areas of hemorrhagic necrosis. In addition to eliciting a positive skin reaction in a previously immune animal, R-E blockade increased the injurious effect of the bacterial toxin, causing death in the majority of animals tested.

Miss Elizabeth Roberts gave technical assistance in this work.

[6] Jaffe, R. H., *Physiol. Rev.*, 1931, **11**, 277.

[7] Gratia, A., and Linz, R., *Ann. Inst. Pasteur*, 1932, **49**, 131.

[8] Giuffrè, T., *Pathologica*, 1937, **29**, 492.

[9] Trizzino, E., and Caffarelli, F., *Riv. di Pat. Sper.*, 1939, **22-23**, 465.

15729

Affinity of Avidin for Certain Analogs of Biotin.

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Wright and Skeggs recently have described a procedure whereby the relative affinity of avidin for biotin and for biotin analogs possessing no microbiological activity readily may be compared.[1] The method was illustrated by a study of the relative affinity of avidin for the available spatial isomers of biotin.

This paper presents additional data on the avidin combinability of certain compounds having some structural similarity to biotin. The compounds studied (I-XIX) may be classified into 4 types: (1) acyclic analogs of desthiobiotin differing from desthiobiotin and its homologs by an opening of the imidazolidone ring (II-VII),[2,*] (2) hydantoin derivatives differing from desthiobiotin homologs by the replacement of the methyl group with an oxygen atom (VIII,IX),* (3) "model" compounds (X-XIII) bearing some relationship to the original pyrimidine structure proposed and later retracted by Kögl *et al.* for α-biotin,[3,4,*] (4) aromatic and cyclic aliphatic derivatives with a urea ring or a urea ring with an ω-carboxy aliphatic side chain (XIV-XIX).[5,†]

The procedure employed[1] involved the addition of avidin to mixtures of biotin and the analog under investigation. The biotin and avidin were used in stoichiometric amounts and the amount of analog varied. In the case of compounds having definite avidin combinability an amount of biotin is available for the growth of *Lactobacillus arabinosus* that is equivalent to the amount of analog taken up by the avidin. The "relative affinity" is then expressed arbitrarily as the ratio of the concentration of analog to biotin at which one-half of the biotin remains free and available for growth of the test organism. It is obvious that the ratio will be low for analogs for which avidin has considerable affinity and high for those analogs that do not combine readily with avidin.

None of the compounds studied had any activity in lieu of biotin for promoting growth of *L. arabinosus*. Similarly, none of the compounds studied, *in the amounts employed,*

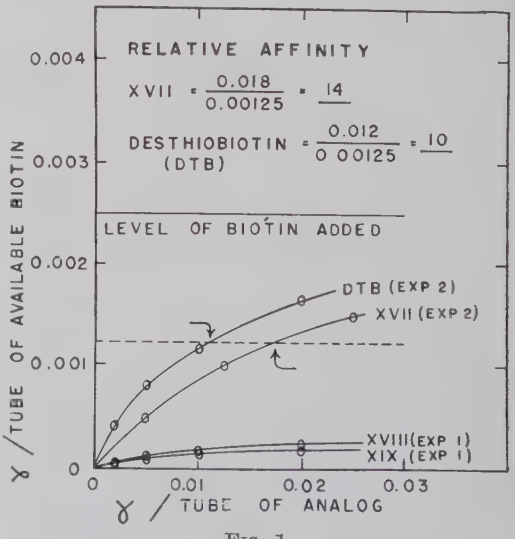


FIG. 1.
The relative affinity of avidin for biotin and biotin analogs.

[1] Wright, L. D., and Skeggs, H. R., *Arch. Biochem.*, 1947, **12**, 27.

[2] Schultz, E. M., in press.

* We are indebted to Dr. E. M. Schultz of these laboratories for compounds II through XIII.

[3] Kögl, F., Verbeek, J. H., Erxleben, H., and Borg, W. A. J., *Z. physiol. Chem.*, 1943, **279**, 121.

[4] Kögl, F., and Borg, W. A. J., *Z. physiol. Chem.*, 1944, **281**, 65.

[5] English, J. P., Clapp, R. C., Cole, Q. P., Halverstad, I. F., Lampen, J. O., and Roblin, R. O., Jr., *J. Am. Chem. Soc.*, 1945, **67**, 295.

† These compounds were supplied through the courtesy of Dr. J. O. Lampen of the Research Laboratories of the American Cyanamid Company.

TABLE I.
The Affinity of Avidin for Certain Analogs of Biotin.

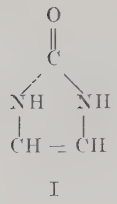
Exp. No.	Biotin, γ	Analog, γ	Avidin, γ	Turbidity
1	0			84
	.00025			141
	.0005			176
	.00075			200
	.0010			220
	.0015			275
	.0025			318
	.0025		20	300
	.0025		50	250
	.0025		100	86
	.0025		200	83
	.0025	.002 XVIII	100	99
	.0025	.005 "	100	106
	.0025	.010 "	100	128
	.0025	.020 "	100	139
	.0025	.002 XIX	100	93
	.0025	.005 "	100	111
	.0025	.010 "	100	125
	.0025	.020 "	100	130
2	0			97
	.00025			163
	.0005			204
	.00075			246
	.0010			260
	.0015			300
	.0025			350
	.0025		20	335
	.0025		50	284
	.0025		100	122
	.0025		200	95
	.0025	.0050 XVII	100	212
	.0025	.0125 "	100	262
	.0025	.0250 "	100	300
	.0025	.050 "	100	330
	.0025	.002 DTB*	100	189
	.0025	.005 "	100	244
	.0025	.010 "	100	276
	.0025	.020 "	100	310

* *dl*-Desthiobiotin.

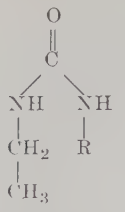
had significant antibiotin activity for the assay organism.

Combinability studies were carried out directly in the microbiologic assay medium by the aseptic addition of avidin just prior to seeding with *L. arabinosus*.

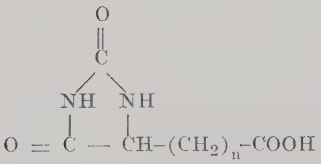
Of the compounds studied only No. XVII, XVIII, and XIX showed definite avidin combinability. Compound No. XVII, which differs from biotin in the replacement of the sulfur atom by a dimethylene group, had an affinity ratio of approximately 14. For reference *d,l*-epi-allobiotin has an affinity ratio of about 3-6[1] while *d,l*-desthiobiotin has an affinity ratio of approximately 10 (Table I, Fig. 1). The specificity of the avidin combinability reaction further is illustrated by the fact that Compound XIX in which the ureylene group is attached to the cyclohexane ring at positions 3 and 4 rather than 2 and 3 with respect to the valeric acid side chain, although showing detectable avidin combinability (Table I, Fig. 1), had an affinity ratio too high for practical measurement. Compound XVIII in which the ureylene group is attached 3,4 to a cyclohexyl ring



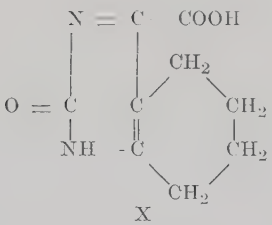
I



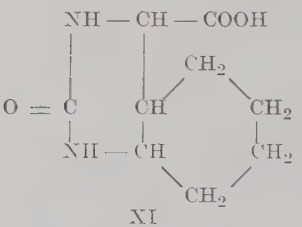
II R = $-CH_2-CH_2-CH_2-CH_3$
III R = $-CH_2-CH_2-CH_2-CH_2-CH_2-COOH$
IV R = $-CH_2-CH_2-CH_2-CH_2-CH_2-CONH_2$
V R = $-CH_2-CH_2-CH_2-CH_2-CH_2-COOC_2H_5$
VI R = $-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-$ COOH
VII R = $-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-$ $COOC_2H_5$



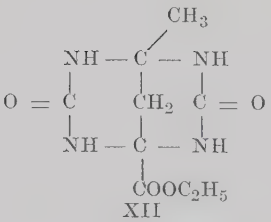
VIII n = 1
IX n = 2



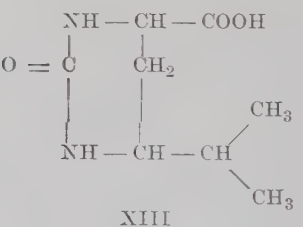
X



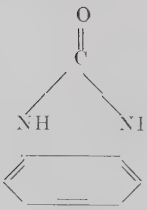
XI



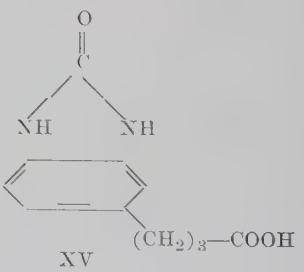
XII



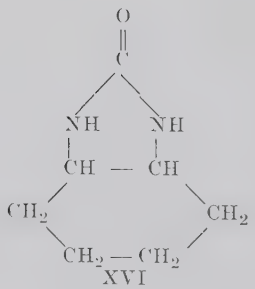
XIII



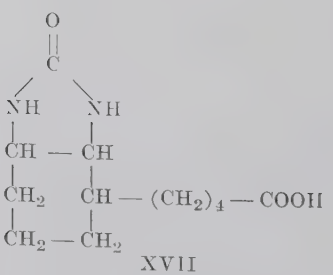
XIV



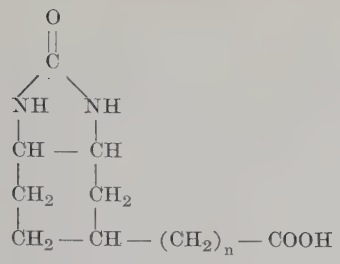
XV



XVI



XVII



XVIII n = 3
XIX n = 4

containing a butyric acid side chain similarly showed definite avidin combinability but had an affinity ratio too high for practical measurement.

The failure of avidin to show significant affinity for the acyclic analogs of desthiobiotin (II-VII) is not unexpected. Opening of the imidazolidone ring would alter considerably the spatial arrangement of the molecule.

The inactivity of the hydantoin derivatives probably is attributable either to the shortness of the side chain in the compounds studied or to the existence of the ring predominantly in an enol form showing little resemblance to the cyclic urea ring of biotin.

While the resemblance of some of the pyrimidine derivatives studied to the original α-biotin formula of Kögl *et al.* is quite remote, Compound XIII is similar to the corresponding desthio derivative. The data do not permit a conclusion as to whether the failure of avidin to show significant affinity for this compound is due to the presence of a pyrimidine rather than an imidazolidone ring or to the absence of an ω-carboxy aliphatic side chain of suitable length.

Winnick, Hofmann, Pilgrim and Axelrod[6] have studied the inhibition of certain oxybiotin derivatives with avidin. It was demonstrated that *dl*-oxybiotin, *dl*-oxybiotin methyl ester and hexahydro-2-oxo-1-furo-(3,4)-imidazole-4-pentanol (the alcohol corresponding to oxybiotin) combined with avidin. Approximately one unit of avidin was required to inactivate each compound for *Saccharomyces cerevisiae*. Cis-3,4-Diamino-2-tetrahydrofuranvaleric acid (oxybiotin diamino carboxylic acid) in which the cyclic urea ring is absent failed to combine with avidin. The procedures employed by Winnick *et al.* are capable of a qualitative interpretation but do not permit a conclusion as to the relative affinity of avidin for biotin in comparison with the affinity for the compounds studied.

Summary. The avidin combinability of a number of compounds having some structural similarity to biotin was investigated. The specificity of the avidin reaction was emphasized by the finding that of the 19 compounds studied avidin possessed significant affinity only for δ-(2,3-ureylene-cyclohexyl)-valeric acid (XVII), γ-(3,4-ureylene-cyclohexyl)-butyric acid (XVIII) and δ-(3,4-ureylene-cyclohexyl)-valeric acid (XIX). The relative affinity ratio for Compound XVII was found to be about 14. Compounds XVIII and XIX, although definitely capable of combining with avidin, had affinity ratios too high for practical measurement.

[6] Winnick, T., Hofmann, K., Pilgrim, F. J., and Axelrod, A. E., *J. Biol. Chem.*, 1945, **161**, 405.

15730

Hepato-Renal Factors in Circulatory Homeostasis.* II. Disappearance of Hepatic Vaso-Depressor Material Following Intravenous Administration.

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(With the technical assistance of S. Rosenfeld, D. Metz, and V. Bergman.)
From the Department of Medicine, Cornell University Medical College, and The New York Hospital, New York City.

Recent *in vivo* and *in vitro* studies[1,2] have established the existence of a hitherto unrecognized vasodepressor principle which enters the blood stream in increasing amounts during the decompensatory stage of hemorrhagic and tourniquet shock. The vasodepressor principle, VDM, was found to be elaborated by the liver and skeletal muscle whenever the blood supply to those tissues was reduced below levels necessary to maintain an oxidative type of metabolism. The quantitative contribution of the liver is considerably greater than that of the skeletal muscle. Maximum blood levels are reached in experimental shock when the hypotension is profound and sufficiently prolonged to result in the development of a state unresponsive to transfusion, the so-called irreversible state.

Once the vasodepressor material appears during profound shock and precipitates the stage of vascular hypo-reactivity, it persists in the blood and is only temporarily diluted when the animal is transfused. The persistence of vasodepressor activity in the blood of shocked animals is in contrast to its transient effect when injected into the blood stream of normal rats for bio-assay. This points to the existence, under normal conditions, of mechanisms for removing this principle from the circulation. *In vitro* incubation experiments[2] have demonstrated that the healthy liver is the only tissue which possesses an enzyme system capable of oxidatively destroying VDM. However, its removal from the blood stream in the living animal may be a more complex phenomenon, with several accessory mechanisms potentially participating in clearing the blood of VDM. Among these are the possible renal excretion of VDM into the urine, the inactivation of VDM by some component of the blood, and the counterbalancing of the vasodepressor effect by the release from the kidney of the oppositely acting vasoexcitor principle, VEM.

A consideration of the mechanisms concerned with the precise regulation of VDM blood levels should weigh the relative contributions of both the liver and kidneys in clearing VDM from the circulation. Experiments were therefore carried out to determine the rate at which endogenous or exogenous VDM was cleared from the blood stream of rabbits with the liver and kidneys intact, and with either the liver or the kidneys, or both, excluded from the circulation.

Methods. The disappearance of *exogenous* VDM was followed by the assay of blood samples taken at intervals following the intravenous injection of a VDM concentrate prepared from anaerobic beef liver (method of preparation used by Dr. A. Mazur to be published elsewhere). The vasodepressor activity of the heparinized plasma samples was measured by the rat meso-appendix technic[3] which uses the reactivity of the terminal arterioles and precapillaries to epinephrine as an index of vasotropic effects. The potency of the liver concentrates was such the 0.1 γ

* Aided by grants from the Josiah Macy, Jr., Foundation and the Eli Lilly Company.

† Research Fellow from the Asuncion Medical School, Asuncion, Paraguay.

1 Zweifach, B. W., Lee, R. E., Hyman, C., and Chambers, R., *Ann. Surg.*, 1944, **120**, 232.

2 Shorr, Ephraim, Zweifach, B. W., and Furchgott, R. F., *Science*, 1945, **102**, 489.

3 Chambers, R., Zweifach, B. W., Lowenstein, B. E., and Lee, R. E., Proc. Soc. Exp. Biol. and Med., 1944, **56**, 127.

to 0.5 γ of VDM (on the basis of nitrogen content) produced a 20- to 30-minute vasodepressor effect in normal, 125 g rats, an effect similar to that produced by the injection of 0.5 cc of plasma from dogs in irreversible hemorrhagic or tourniquet shock.

Four types of experimental animals were used:

(1) *Controls with liver and kidneys in the circulation.* Normal rabbits, and rabbits which had been partially eviscerated according to a technic described by Engel,[4] leaving intact the arterial blood supply to the liver via the hepatic artery. (2) *Arenal animals.* The renal artery and vein were tied off to exclude the kidneys from the circulation. (3) *Hepatectomized animals.* Complete evisceration was carried out so as to exclude the liver from the circulation but leave the kidneys intact. (4) *Hepatectomized-arenal animals.* Complete evisceration excluding both the liver and kidney from the circulation.

A series of 16 rabbits, anesthetized with Seconal (25 mg per kg) were used in this group of experiments. Following the operative procedures the rabbits were transfused with 20 to 40 cc of whole blood or 5% albumin until the blood pressure became stabilized in the 90 to 100 mm Hg range. Control blood samples gave a neutral bio-assay. Two to 3 cc of the liver VDM concentrate were then injected intravenously and samples of femoral vein blood (2 to 3 cc) were removed within one to 2 minutes and at 10-minute intervals thereafter, until one hour had elapsed. For assay, about 0.5 cc of plasma was injected intravenously into normal rats in which the meso-appendix had been exposed for microscopic observation. The amount of VDM present in the injected blood samples was determined by noting the length of time that the terminal arterioles of the test rat remained unresponsive to epinephrine. The details of this method of assay are given in previous publications of Chambers and Zweifach.[1,3]

The disappearance of *endogenous* VDM was studied in the partially eviscerated rabbit preparation in which the hepatic artery was left as the sole source of blood supply to the liver. Temporary hepatic anoxia was produced by placing a clamp on the hepatic artery for varying periods of time, for the purpose of inducing VDM formation in the liver. The re-establishment of the hepatic circulation after a 45- to 90-minute period of liver anoxia led to the appearance of VDM in the blood stream. The disappearance of endogenous VDM was followed in 5 rabbits with either both the liver and kidneys in the circulation, or with the kidneys excluded.

Results. A. *The Disappearance of Exogenous VDM.* 1. *Controls, Liver and Kidneys in Circulation.* The most rapid removal of VDM from the blood was found in the control series of animals in which both the liver and kidneys were intact. As shown in Fig. 1, blood taken one to 2 minutes after injection of vasodepressor concentrate gave a 25- to 35-minute VDM bio-assay and then rapidly fell off in activity, becoming neutral within 30 to 40 minutes. The higher the initial blood concentration of VDM, the longer was the period required for the animal to clear the blood of this principle.

2. *Arenal Animals.* When the kidneys were tied off and the liver allowed to remain in the circulation, the VDM blood levels showed a more gradual decline, the depressor principle remaining in the blood somewhat longer than in control animals. In Fig. 2 it can be seen that the VDM was not completely cleared from the blood until 40 to 50 minutes had elapsed. The gradual disappearance of VDM in these animals closely resembled the progressive inactivation of VDM which occurred when liver slices were incubated aerobically *in vitro* with similar vasodepressor solutions.[2]

3. *Hepatectomized Animals.* On the basis of *in vitro* inactivation experiments in which only the liver could inactivate VDM, it might be predicted that exclusion of the liver from the circulation should result in the accumulation of VDM in the blood. This, however, was not the case; in eviscerated-hepatectomized animals with an intact kidney circula-

[4] Engel, F. C., Harrison, H. C., and Long, C. N. H., *J. Exp. Med.*, 1944, **79**, 9.

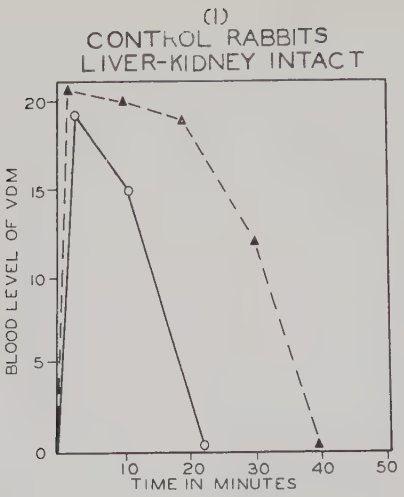

FIG. 1. *Control Rabbits*—includes a normal rabbit (○) and a partially eviscerated rabbit with the hepatic artery to the liver intact and with the kidneys in the circulation (▲).

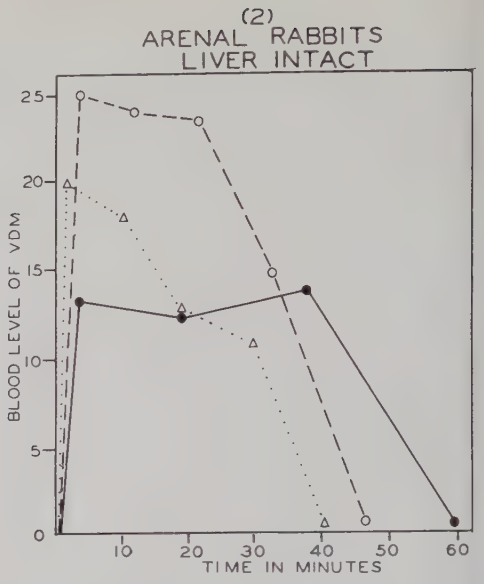

FIG. 2. *Arenal Rabbits*—includes 2 hepatic artery preparation animals (△, ○) and one normal animal (●).

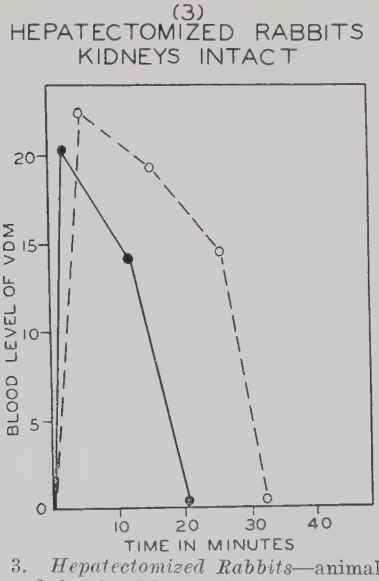

FIG. 3. *Hepatectomized Rabbits*—animals were eviscerated, leaving kidneys in circulation.

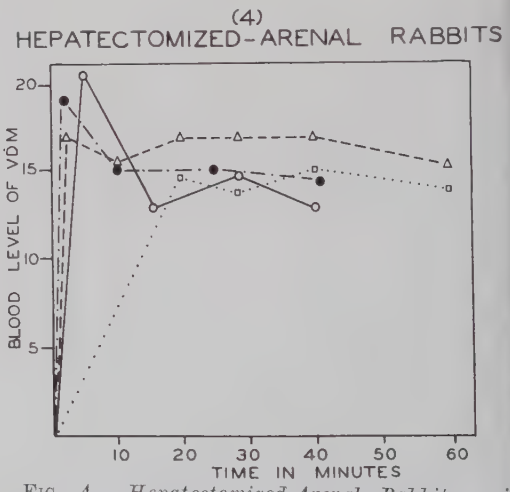

FIG. 4. *Hepatectomized-Arenal Rabbits*—animals were hepatectomized and renal pedicle tied off.

FIG. 1, 2, 3, 4.

Graphs of VDM blood levels following the intravenous injection of a vasodepressor concentrate prepared from anaerobic beef liver. The amounts of vasodepressor material in the blood were determined by the rat meso-appendix test and are expressed along the ordinate axis in terms of the number of minutes that the arterioles and precapillaries remained unresponsive to epinephrine.

tion, VDM disappeared from the blood within 30 to 40 minutes after its intravenous administration (Fig. 3). Since kidney tissue cannot inactivate VDM, another renal mechanism, presumably excretion, appeared to be the means for its removal under these conditions. This inference was confirmed by experiments on the dog in which urine samples were collected and tested for vasodepressor activity. A dog was hepatectomized and the kidneys left in the circulation. Blood and urine samples were taken following the

intravenous injection of the liver vasodepressor concentrate, the urine being collected from the bladder by means of an in-dwelling catheter. In order to eliminate the undesirable effects on the rat test of the concentrations of urea and electrolytes in the urine, it was dialyzed against distilled water overnight in the refrigerator before being tested. Considerable quantities of VDM were found in the urine after the intravenous injection of VDM, whereas the control urine showed only a mild vasodepressor activity. The excretion into human urine of a principle having similar vascular depressor effects has recently been demonstrated in this laboratory by Dr. R. F. Furchgott.

The difference between the controls and the arenal animals points to the participation of the kidney in the removal of VDM from the blood. Additional evidence for such participation was obtained from experiments in which meso-appendix assays were made on both normal rats and arenal rats using the same VDM sample. When the kidneys were tied off, the depressor effects of VDM on the peripheral vessels persisted for longer periods than in normal rats.

Blalock[5] and more recently Van Slyke and co-workers[6] have shown that renal blood flow is reduced to negligible levels in profound shock. The loss of the renal-excretory route for VDM disposal undoubtedly serves as an additional factor to perpetuate the blood vasodepressor activity and thereby contributes to the development of an irreversible state.

4. *Hepatectomized-Arenal Animals.* The kidneys and the liver appear to be the only tissues concerned with the removal of VDM from the blood. When both these organs were excluded from the circulation of the rabbit, the blood level of VDM remained significantly unaffected over a period of an hour, the duration of the experiment (Fig. 4).

The persistence of VDM activity in the blood in the absence of the liver and kidney indicated that the blood *per se* was not concerned with VDM inactivation. This was further confirmed by the observation that plasma or serum suffered little or no loss in VDM activity during aerobic incubation at 37.5°C for 2 to 3 hours.

Previous *in vivo* experiments[2] have demonstrated that the capacity of the liver to inactivate VDM is progressively impaired in shock by the hypoxic state in the organ. In the present series a similar situation was encountered in 3 rabbits which had developed profound hypotension for about 60 minutes following accidental blood-loss during the evisceration procedure. The rabbits were then transfused with large amounts of whole blood until they had returned to normal blood pressure levels. In one animal the kidneys were then tied off and the VDM concentrate injected into the blood stream. No significant removal of VDM occurred and the animal went into profound shock following the removal of only 8 cc of blood for testing purposes. In the other 2 rabbits the kidneys were left in the circulation. Despite the presence of the kidneys, the intravenous administration of VDM was likewise followed by its persistence in the circulation and by a progressive fall of blood pressure to shock levels.

B. *The Disappearance of Endogenous VDM.* These studies were made on a special preparation in which the gastro-intestinal tract was completely removed depriving the liver of its portal blood supply but leaving the hepatic artery intact. Long and co-workers[6] have found that the liver in such animals is maintained in a healthy condition for at least 4 to 5 hours, the duration of their observation. In our own studies, the aerobic state of the liver metabolism under these circumstances is indicated by the absence of VDM in the blood. When the hepatic artery was occluded for 60 minutes and then released, considerable amounts of VDM appeared in the blood stream within 15 minutes. In animals with an intact renal circulation, the VDM disappeared from the blood within 60 minutes after release of the hepatic clamp. When the kidneys were tied

[5] Blalock, A., and Levy, S. E., *Am. J. Physiol.*, 1937, **118**, 734.

[6] Van Slyke, D. D., and co-workers, *Conference on Hemorrhage, Ann. N. Y. Acad. Sci.*, 1946.

off before releasing the clamp on the hepatic artery, large amounts of VDM accumulated in the blood. The blood pressure fell to shock levels within 45 minutes. Evidently, the VDM inactivating mechanism of the liver had been damaged by the period of anaerobiosis and was no longer capable of inactivating the VDM in the blood.

Summary. *In vitro* studies have shown that the vasodepressor principle, which appears during the hypo-reactive stage of shock and results from the anoxia of liver and skeletal muscles, can be inactivated under aerobic conditions by healthy liver slices. The present study was concerned with the mechanisms by which endogenous and exogenous VDM are removed from the circulation of the living animal. The exogenous VDM was concentrated and purified from saline extracts of anaerobic beef liver. The endogenous VDM was released into the blood following liver anoxia produced by temporary occlusion of the hepatic artery in a partially eviscerated preparation. Two mechanisms for VDM removal were revealed: (1) its inactivation by the healthy liver; (2) its excretion into the urine by the normal kidney. A preliminary period of hepatic anoxia rendered the liver incapable of inactivating VDM *in vivo,* presumably through anoxic damage to the hepatic enzyme system for this function. This situation is analogous to the progressive impairment of the VDM inactivating mechanism in the liver which develops during the course of shock and which is considered responsible for the perpetuation of the hypo-reactive state of the peripheral vascular system. The loss of the renal excretory function for VDM during hypo-reactive shock deprives the animal of an important means of clearing the blood of VDM and thereby aiding in liberating the vascular bed from this decompensatory vasotropic principle.

15731

Functional Components of the Greater Superficial Petrosal Nerve.

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Larsell and Fenton[1] early emphasized the importance and pointed out the inadequacies of our knowledge of the greater superficial petrosal nerve. Later Chorobski and Penfield[2] added emphasis to the subject by demonstrating that the nerve is an important pathway for vasodilator fibers of the seventh cranial nerve to the pial arteries. More recently Foley and DuBois[3] experimentally separated the functional components of the nerve in a limited number of animals and described its varieties of sensory and motor fibers. Numerative studies on the functional components of the greater superficial petrosal nerve, in a satisfactory number of animals, are needed to complete our knowledge of the composition of the nerve.

Methods. The motor nerve fibers of the greater superficial petrosal nerve of the right side of 9 cats and one dog were eliminated by cutting the rootlets of the facial nerve within the cranium. This operation produced a nerve that, aside from an occasional easily identifiable[3] sympathetic fascicle, consisted only of sensory axons derived from the geniculate ganglion of the seventh cranial nerve.

After allowing a minimum of 14 days for disappearance of the motor axons, the right degenerated and left normal nerves of each

1 Larsell, O., and Fenton, R. A., *Laryngoscope,* 1928, **38**, 371.

2 Chorobski, J., and Penfield, W., *Arch. Neur. and Psychiat.,* 1932, **28**, 1257.

3 Foley, J. O., and DuBois, F. S., *J. Comp. Neur.,* 1943, **79**, 79.

TABLE I. The Greater Superficial Petrosal Nerve.

Animal No.	Surgical procedure	Operated (right) side: Total axons	Ratio* total motor axons	Myelinated axons	Ratio* myelinated motor axons	Unmyelinated axons	Ratio* unmyelinated motor axons	Unoperated (left) side: Total axons	Myelinated axons	Unmyelinated axons
Cat 126†	Intracranial sect. roots	795s-1051m	2.07	706s-1137m	1.61	89s-514m	5.77	2446	1843	603
Cat 2†	Intrapontine sect. roots	845s-1872m	2.21	424s-1491m	3.50	421s-381m	0.90	2717	1915	802
Cat 5†	Intrapontine sect. roots	491s-1527m	3.11	473s-1070m	2.26	18s-457m	25.11	2018	1543	475
Cat 127‡	Intracranial sect. roots	800s- 840m	1.05	580s 445m	0.76	220s-395m	1.79	1640	1025	615
Cat 161‡	Intracranial sect. roots	608s-1721m	2.83	516s-1002m	1.96	92s-719m	7.81	2329	1518	811
Cat 162‡	Intracranial sect. roots	366s-2228m	6.08	252s-1678m	6.65	114s-550m	4.81	2594	1930	664
Cat 163‡	Intracranial sect. roots	578s-1565m	2.70	530s-1100m	2.07	48s-465m	9.68	2143	1630	513
Cat 164‡	Intracranial sect. roots	369s 1770m	4.79	272s-1709m	6.17	97s- 61m	0.62	2139	1981	158
Cat 170†	Intracranial sect. roots	525s-1192m	2.27	400s- 840m	2.10	125s-352m	2.81	1717	1240	477
	Axon averages for all nerves of cat	597s-1596m	2.67	461s 1164m	2.52	136s-432m	3.17	2193	1625	568
Dog 1†	Intrapontine sect. roots	960s-2527m	2.63	664s-1635m	2.45	296s-892m	3.01	3487	2299	1188

* The single number represents the ratio of motor axons as derived by dividing the number of motor fibers by the number of sensory fibers.
s—Sensory nerve fibers.
m—Motor nerve fibers.
† Axons right and left nerves stained with pyridine silver technic.
‡ Axons right and left nerves stained with Bodian technic.

animal were secured and processed for enumeration of the myelinated and unmyelinated nerve fibers with a variant of either the pyridine silver[4] or Bodian technic.[4]

Complete counts were made in selected cross sections[4] of the sensory axons which remained in the degenerated nerves, whereas, the combined sensory and motor nerve fibers of the nerves of the unoperated sides were estimated in transverse sections by means of the strip method.[5] The number of motor axons that were present in the deefferented nerves before operation was approximated by subtracting the sensory fibers which remained in the degenerated nerve from the combined motor and sensory axons which were enumerated in the control nerve.

The total number of nerve fibers and the unmyelinated axons in both the degenerated and normal nerves was counted or estimated. The number of myelinated nerve fibers was then derived by subtracting the unmyelinated type of axons from the total number of nerve fibers.

Results. The normal greater superficial petrosal nerve of the cat (Table I, unoperated side) contains from 1640 (cat 127) to 2717 (cat 2) combined sensory and motor axons. The myelinated sensory and motor axons of the intact petrosal nerve vary from 1025 (cat 127) to 1981 (cat 164), whereas only 158 (cat 164) to 811 (cat 161) are without myelin sheaths. The single normal specimen of the petrosal nerve of the dog (dog 1, Table I) has 3487 axons which are divided into 2299 myelinated and 1188 unmyelinated nerve fibers.

Of the deefferented nerves of the cat which contain only sensory fibers (s, Table I), the total number of sensory axons varies from 366 (cat 162) to 845 (cat 2). The myelinated type of sensory nerve fiber ranges from 252 (cat 162) to 706 (cat 126) and the unmyelinated type of sensory axon varies from 18 (cat 5) to 421 (cat 2). The deefferented petrosal nerve of the dog had

[4] Foley, J. O., Pepper, H. R., and Kessler, W. H., *J. Comp. Neur.*, 1946, **85**, 141.

[5] Davenport, H. A., and Barnes, J. F., *Stain Tech.*, 1937, **10**, 139.

960 sensory axons and these included 664 with myelin sheaths and 296 that lacked myelin sheaths.

The greater superficial petrosal nerve of the cat contains from 840 (cat 127) to 2228 (cat 162) motor axons (m, Table I). The myelinated type of motor axon ranges from 445 (cat 127) to 1709 (cat 164). Fewer unmyelinated motor axons, 61 (cat 164) to 719 (cat 161), were found in the greater superficial petrosal nerves of the 9 cats under consideration. The petrosal nerve of the dog had 2527 motor nerve fibers and of these 1635 were myelinated and 892 unmyelinated.

It is apparent from these data and the averages listed in Table I that the motor axons are the predominant functional type of nerve fiber in the greater superficial petrosal nerve of the cat and dog. Although the proportion of total sensory to total motor axons may approach a 1:1 ratio as in cat 127, Table I, where there are 1.05 motor axons for each sensory nerve fiber; yet, on the other hand, there may be as many as 6.08 motor nerve fibers for each sensory axon (cat 162). A similar spread in the ratio between sensory and motor axons occurs when only the myelinated varieties are considered; compare cat 127 with cat 162. There is a greater spread in the ratio of sensory to motor axons when only the unmyelinated variety of axon is considered. There may be a few more sensory unmyelinated nerve fibers (cat 2, 164) or a pronounced excess of unmyelinated motor axons (cat 5). The average numbers of sensory and motor axons of all types (Table I, averages*) show essentially the same proportionate deviation to the motor side. In the single specimen of the dog, the ratio of sensory to motor axons of all varieties approaches a magnitude of 1:3 (Table I, dog 1*).

Discussion. The only comprehensive work on the number of nerve fibers in the normal greater superficial petrosal nerve is that of Van Buskirk.[6] Van Buskirk listed 252 to 2268 nerve fibers of all types in 13 nerves of the cat, 654 to 3947 axons in 39 counts of the petrosal nerve of the dog and 862 to 1687 nerve fibers in the greater superficial petrosal nerve of man.

The total number of axons (3487) reported by the author for the normal greater superficial petrosal nerve of the dog (Table I, unoperated side) fits into the range in number of axons which Van Buskirk records for the greater superficial petrosal nerve of the dog.

The enumerations in the intact petrosal nerve of the cat which have been made by the writer (Table I, unoperated side) approximate the maximum number reported by Van Buskirk. In no instance was any count as low as the minimum figure recorded by Van Buskirk. Most of the axon values reported herein for the intact greater superficial petrosal nerve, however, were within 10% of the maximum number given by Van Buskirk for the normal petrosal nerve of the cat. Deviations of this percentile value represent a reasonable check. The explanation for the difference in the minimal and maximal values reported by Van Buskirk and the writer is not apparent; it may be due to normal variation in the nerves of different animals, to the difference in the quality of the silver preparations used by 2 different workers, or it may be a consequence of the different methods used in the 2 investigations for estimating the number of nerve fibers.

There is a poverty of experimentally derived data on the ratio of sensory to motor fibers in the greater superficial petrosal nerve. Of recent interest are the experimental results of Schimert[7] and Kure and Sano.[8] Schimert believes that the nerve contains very few if any sensory axons; since he was unable to see any myelinated axons in the greater superficial petrosal nerve of the rat and but few axons in the greater superficial petrosal nerve of the cat after cutting the facial nerve proximally in these animals. Kure and Sano report that the

[6] Van Buskirk, C., *J. Comp. Neur.*, 1945, **82**, 303.

[7] Schimert, J., *Z. f. mikr. anat. Forsch.*, 1936, **39**, 35.

[8] Kure, K., and Sano, T., *Z. f. Zellforsch. u. mikr. Anat.*, 1935, **23**, 495.

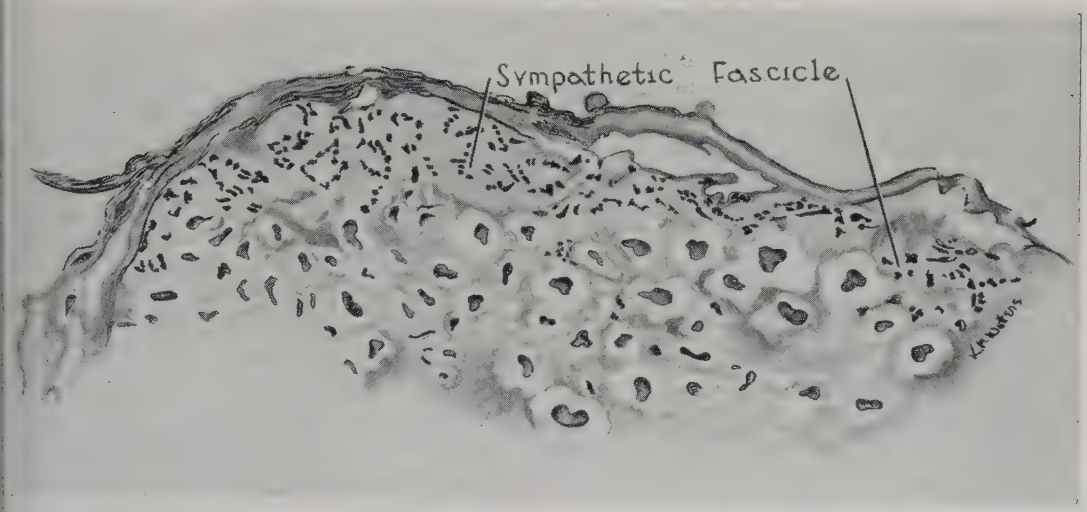

FIG. 1.
From a cross section of the greater superficial petrosal nerve of the cat which has been freed of motor fibers of seventh nerve origin. Attention is directed particularly to the peripherally situated fascicle of sympathetic nerve fibers. Pyridine silver stain.

greater superficial petrosal nerve of the dog contains but few myelinated axons either before (276) or after (145) deletion of its motor nerve fibers by intracranial section of the facial nerve. The observations of these authors are not in accord with the experimental findings of the writer; for, not only do the normal greater superficial petrosal nerves of the cat and dog contain large numbers of myelinated nerve fibers but a sizeable number of myelinated sensory fibers remains in the nerves of both animals (s, Table I, operated side) after the motor fibers have been eliminated.

In our original report[3] on the greater superficial petrosal nerve of the cat, it was stated that roughly 62% of the axons of the nerve was motor. Now it is evident that, although the nerves of some cats (cat 126) may contain about this proportion of motor axons, there may be less (cat 127, 51%) or more (cat 162, 85%) motor nerve fibers in the greater superficial petrosal nerve of the cat. The average number of motor nerve fibers in the nerve of the cat (Table I) constitutes about 72% of the total number of nervus intermedius axons in the greater superficial petrosal nerve.

Finally, the proposed ratios (Table I*) between sensory and motor fibers may not be an exact representation of the ratios of sensory to motor axons which are exclusively of seventh nerve origin. A relatively small number of axons in both the operated and intact nerves may be sympathetic in origin; since, as reported earlier,[3] one or more small fascicles of postganglionic sympathetic motor axons may enter the greater superficial petrosal nerve at or just distal to its origin from the geniculate ganglion. These fascicles (Fig. 1) can be readily identified by one familiar with the anatomy of postganglionic bundles and the axons of these aggregations have been omitted in the enumerations which are recorded in Table I. On the other hand, some of the sympathetic nerve fibers may scatter from these bundles[9] among the intermedius axons of the greater superficial petrosal nerve. Such intermingling would influence the enumerations for total and unmyelinated axons but would not affect the ratios of myelinated sensory and motor nerve fibers since practically all postganglionic sympathetic axons are without demonstrable myelin sheaths.[9]

Summary. The normal greater superficial petrosal nerve of the cat averages 2193 axons and of these, 1625 are myelinated and 568

[9] Foley, J. O., *J. Comp. Neur.*, 1945, **82**, 77.

are without myelin sheaths. A single intact nerve of the dog held 3487 nerve fibers, which were divided into 2299 myelinated and 1188 unmyelinated axons.

An average of 597 sensory nerve fibers remains in the greater superficial petrosal nerve of the cat after its motor axons have been eliminated. Of this average number of sensory nerve fibers, 461 are myelinated and 136 are unmyelinated. The deefferented petrosal nerve of one dog contained 960 sensory axons and 664 of these were myelinated while 296 were without myelin sheaths.

It is estimated that the greater superficial petrosal nerve of the cat has an average of approximately 1596 motor nerve fibers and these are divided into 1164 myelinated and 432 unmyelinated nerve fibers. The greater superficial petrosal nerve of the dog contained 2527 motor axons and 1635 of these were myelinated and 892 were unmyelinated.

It is evident, therefore, that the majority of the axons of the greater superficial petrosal nerve are motor nerve fibers, there being an average of 2 plus motor fibers for each sensory axon.

15732

Employment of the Embryonic Duck Heart for the Detection of Minute Amounts of a Digitalis Glycoside* (Lanatoside C).

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Exact quantitative information concerning the absorption and excretion (or destruction) of digitalis in the human body is still to be obtained. Such knowledge has not been gained primarily because of the absence of any test sufficiently sensitive to detect the relatively minute amounts of digitalis which must be present in the tissues of patients receiving digitalis. The use of the frog, cat and pigeon for the assay of digitalis has been limited to the quantitation of relatively large quantities of the drug and cannot be utilized for the demonstration of digitalis if the latter is present in but fractions of a microgram.

Pickering[1] was the first to employ the embryonic chick heart for the detection of digitalis. Using embryos between 60-75 hours of age, he demonstrated the striking similarity of such hearts to that of the adult mammal, especially in regard to their response to digitalin and strophanthin. He found that the embryonic chick heart would cease beating after the injection of 0.012 mg of digitalis *in situ*. Lagen and Sampson[2] applied digifoline directly upon the embryonic heart of the chick and were able to demonstrate an electrocardiographic effect after such a procedure. Hall[3] also employed the embryonic chick heart for the assay of crude digitalis. Paff[4-6] dissected the heart from the chick embryo and immersed it in the solution containing the drug to be tested. Using the occurrence of an arrhythmia as a test for the presence of both ouabain and

* Aided by a grant from the Dazian Foundation for Medical Research and by a grant from the Sandoz Chemical Works, Inc.

1 Pickering, J. W., *J. Physiol.*, 1893, **14**, 383.

2 Lagen, J. B., and Sampson, J. J., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 735.

3 Hall, E. M., *Am. J. Pharm.*, 1932, **104**, 310.

4 Paff, G. H., *J. Pharm. and Exp. Therap.*, 1932, **69**, 311.

5 Paff, G. H., and Johnson, J. R., *Am. J. Physiol.*, 1938, **126**, 753.

6 Paff, G. H., *J. Pharm. and Exp. Therap.*, 1940, **70**, 235.

digitoxin, he believed he could detect the presence of as little as 1/100,000 of ouabain and 0.001 mg per cc of digitoxin. He found however, that there was a wide fluctuation in the time of appearance of the arrhythmia at any given concentration of the drug tested.

Although the embryonic chick heart appeared to be suitable for employment in the detection of digitalis, it still was not sufficiently sensitive to give much promise as an aid in the quantitation of the concentration of digitalis in the human body. For this reason, the embryonic duck heart was studied and compared to the chick heart in particular respect to their sensitivity to the digitalis glycoside, Lanatoside C. The results of this study are given in this communication.

Methods. A. The Embryonic Duck Heart. Supposedly fertile duck eggs (White Peking China) were incubated for 88-92 hours at 39°C. They then were transilluminated and the position of the embryo marked on the shell. The shells were then partially removed until the embryo and its vascular sinus were in full view. After the diameter of the sinus had been measured, the total embryo was removed with a sharp scissors and placed in a slide well containing Tyrode's solution. Under a dissecting microscope (12×), the heart was removed from the embryo by means of dissecting, cataract knives. At this stage of development, the heart bulges from the body, just caudal to the head and easily can be identified. It also apparently represents one of the most adhesive, solid tissues of the embryo for it may be separated in its entirety by simple teasing of embryonic tissue lying adjacent to it. After separation, the heart is floated gently upon a small spatula and transferred to a second slide well containing 0.1 cc of the solution to be tested. This second slide rests on the stage of a compound microscope which is in a box automatically maintained at 33°C, by means of an electric lamp of 30 watts in circuit with a mercury thermostat and vacuum tube relay switch. After this second transfer, the heart is observed through the microscope (30×) and (1) strength and rate of contraction, (2) occurrence of an arrhythmia and (3) duration of beating are noted.

It was found after preliminary studies that those hearts of embryos contained in vascular sinuses measuring from 20-30 mm in diameter were most suitable for the detection of the digitalis glycoside employed.

Lanatoside C, the glycoside of digitalis lanata, was used in all experiments. An ampule containing 0.2 mg per cc was opened for each day's experiments. Dilutions of this drug varying from 0.001 to 0.00001 mg per cc were studied, after a control series of embryonic hearts were immersed in simple Tyrode's solution and studied.

B. *The Embryonic Chick Heart.* Embryonic chick hearts were obtained and studied in the same manner except that (1) they were incubated only 66 hours at 38.5°C and (2) no embryo having a sinus diameter greater than 30 mm was used.

Results. A. *The Embryonic Duck Heart.* The embryonic duck heart was found to be extraordinarily sensitive to the digitalis glycoside employed. The first indication of this sensitivity was an acceleration and an intensification in the vigor of cardiac contractions. Then, just prior to the occurrence of auriculoventricular block (which was used as the actual indicator for the presence of digitalis) the heart was observed to contract irregularly, very rapidly, jerkily and weakly. The auriculoventricular block always began as partial A-V block and progressed to complete block. Concomitant with the progression of the block, the irregularity of the rate of beating became more manifest. Finally, if sufficient concentrations of digitalis were used, the heart stopped beating in systole.

Thus it was found (Table I A) that when 23 embryonic duck hearts were placed in 0.1 cc of a solution containing 0.001 mg of the glycoside per cc, each began to accelerate and all began to demonstrate auriculoventricular block after an average period of 12 minutes (range: 6 to 28 minutes). They ceased to beat after an average period of 37 minutes (range: 29 to 62 minutes). Approximately the same phenomena were observed when 15 hearts were placed in 0.1 cc of a

TABLE I.
Effect of Digitalis on the Embryonic Duck and Chick Heart.

Digitalis (mg/cc)	Avg sinus diameter (mm)	Hearts (No.)	A-V block (No.)	Onset-A-V block (min.)	Duration of beating (min.)	Avg rate	Type of Contractions
			A. Embryonic Duck Heart.				
0 (control)	30	15	0	—	>60	59	Moderate
.001	31	23	23	12	37	85	Vigorous
.0005	33	15	15	15	42	82	,,
.0001	29	15	15	35	54	76	,,
.00005	32	18	14	39	>60	73	,,
.00001	28	16	0	—	>60	61	Moderate
			B. Embryonic Chick Heart.				
0 (control)	27	10	0	—	>60	76	,,
.001	21	25	21	15	41	96	Vigorous
.0005	23	14	10	18	>60	96	,,
.0001	17	10	0	—	>60	82	Moderate

solution containing 0.0005 mg of glycoside per cc (Table I A) except that onset of auriculoventricular block occurred somewhat later (average: 15 minutes) and the hearts continued to beat somewhat longer (average: 42 minutes). When 15 hearts were placed in a solution containing 0.0001 mg of glycoside per cc, auriculoventricular block occurred in all of them (Table I A) but the average time of onset was 35 minutes. These latter hearts also continued to contract for an average period of 54 minutes (range: 43 to 71 minutes). Auriculoventricular block occurred in 14 of 18 hearts (78%) placed in a solution containing 0.00005 mg of glycoside per cc after an average period of 39 minutes (range: 24 to 68 minutes). The longevity of these hearts was not affected however by the concentration of glycoside employed. No auriculoventricular block occurred in any of 16 embryonic hearts immersed in a solution containing 0.00001 mg of glycoside per cc (Table I A).

B. *The Embryonic Chick Heart.* As can be seen by inspection of Table I B, the action of the digitalis glycoside on the embryonic chick heart was similar to its effect upon the duck heart except that the former type of heart was much less sensitive to the drug. Thus (Table I B) whereas a concentration of as little as 0.00005 mg of glycoside per cc could be detected by the use of the duck heart preparation, only 0.0005 mg per cc could be detected by the chick heart. Furthermore, even as much of the digitalis glycoside as 0.001 mg per cc could not be invariably detected by using the chick heart method.

Discussion. The above results indicated that the embryonic duck heart was far more sensitive to the digitalis glycoside employed than the embryonic chick heart. By the former's employment, 0.1 cc of a one to 20 millionth dilution of Lanatoside C. (0.000005 mg) could be detected. It is believed that this represents the most sensitive indicator for the presence of a digitalis glycoside yet described.

The embryonic heart of the duck also has certain advantages for its use in the study of cardiac physiology. Pickering[1] earlier stressed the similarity of the chick heart to the mammalian heart and it is most likely that the duck heart also has the same similarities. There are no nervous elements in the embryonic heart at this stage,[1] a state of affairs which allows the study of cardiac musculature alone. Furthermore, the ability of this type of heart to contract regularly over a period of at least 60 minutes in very small quantities of fluid, allows the possibility of testing samples, meager in amount. Finally, because of the relative paucity of tissue composing the heart, more opportunity is allowed for changing the ionic milieu of the cardiac cells (when desired) than might be possible were an adult mammalian heart the subject of experimentation.

Conclusion. The embryonic duck heart, similar to the embryonic chick heart was found to exhibit sensitivity to the action of a digitalis glycoside as characterized by alteration in rate, rhythm and force of contraction. Moreover the embryonic duck heart was able to be used to detect as little as one two-hundredth of a microgram of the digitalis glycoside (Lanatoside C.) This is thought to represent the most sensitive indicator now available for the presence of a digitalis glycoside.

15733 P

Isolation of Pleuropneumonia-like Organisms from Pathological Specimens with the Aid of Penicillin.*

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Pleuropneumonia-like organisms (P.P.L.O.) were isolated from the genito-urinary tract of human patients and in rare cases from other locations.[1-5] Such organisms are often present in the vagina or cervix without apparent connection with any disease. In males they occur only in pathological conditions, and, according to observations collected in the last few years, they play a noticeable role as causative agents in urethritis, prostatitis and cystitis. They have been isolated from cases of severe cystitis which were repeatedly negative for the usual bacteria. Infection of the genito-urinary tract was complicated in about 30% of the cases with acute polyarthritis. It is essential in studying the role of these organisms in human disease that the methods used for their culture and identification be free from error.

The presence of P.P.L.O. can be proven only by cultivation. They grow on media enriched with human or animal serum or with ascitic fluid. Large colonies (0.1-0.3 mm) can be identified with the low power of the microscope. If the colonies remain small (.01-0.1 mm) as often happens in cultures from pathological specimens, they cannot be identified by this method. The agar fixation method which Klieneberger[4] and Salaman[5] used utilizes the impression left by the culture on a coverslip. Small colonies do not adhere to the glass and can not be recognized. This method is subject to error even in the case of large colonies. They are identified by the round bodies which develop on the surface layer and it is not unusual for bacteria to produce similar large bodies. The most reliable method for the identification of the P.P.L.O. is the use of stained agar preparations.[6] A square of the agar culture is cut out and is then covered with a coverslip carrying the stain. The colonies are present in their entirety in the preparations and can be identified with certainty.

Demonstration of P.P.L.O. is usually not possible in the presence of abundant bacterial growth. The P.P.L.O. are resistant to sulfonamides and to penicillin both of which were recommended to suppress bacterial growth. Salaman used penicillin cups for this purpose.[5] The author can confirm the

* The expenses of this investigation have been defrayed in part by a grant from the Commonwealth Fund.

This is publication No. 91 of the Robert W. Lovett Memorial Foundation for the study of crippling disease.

1 Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 470.

2 Dienes, L., and Smith, W. E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 99.

3 Beveridge, W. J. B., *Med. J. Australia*, 1943, **2**, 479.

4 Klieneberger-Nobel, E., *The Lancet*, 1945, **2**, 46.

5 Salaman, M. H., and collaborators, *J. Path. Bact.*, 1946, **58**, 31.

6 Dienes, L., *J. Inf. Diseases*, 1939, **65**, 24.

effectiveness of penicillin, but without necessary precautions its use leads to error and will confuse the study of P.P.L.O.

One source of error is that in the area of inhibition, the colonies may remain very small, and the organisms may swell up into large round bodies. They can not be distinguished in an impression preparation from colonies of P.P.L.O. It is probable that the colonies which Salaman identified as mixed colonies of Gonococci and P.P.L.O. were such altered Gonococcus colonies. The author has never seen P.P.L.O. and Gonococcus together in cultures from male patients.

This source of error can be eliminated by using stained agar preparations in which penicillin does not interfere with the identification of P.P.L.O.

The second source of error is the fact that P.P.L.O. can develop from bacteria under the influence of penicillin. It has been shown in another paper that this occurs in pure culture of *H. influenzae*.[7] From a series of 14 throat cultures which were recently studied, P.P.L.O. developed in 10 in the vicinity of the penicillin cups. The distribution of the colonies indicated that they were produced by the penicillin. They were situated in the area of inhibition and were absent in the area not exposed to penicillin. Colonies of P.P.L.O. never develop in throat cultures without the use of penicillin, while they grow very well together with bacteria in cultures from the genito-urinary tract.

According to these observations, penicillin can be used only to screen the specimens for the presence of P.P.L.O. In order to be sure that they are present in the specimens and are not produced from bacteria, their colonies must be seen also in cultures not treated with penicillin. The P.P.L. strains isolated from human patients form a heterologous group, and it is important to distinguish those which are connected with bacteria from those which, like the animal pathogens, do not show such connection.

Summary. Penicillin is of considerable help in isolating pleuropneumonia-like organisms from specimens contaminated with bacteria. Penicillin alters the colonies of certain bacteria in such a manner that in impression preparations they become indistinguishable from pleuropneumonia-like organisms. Furthermore penicillin may induce the growth of pleuropneumonia-like organisms from bacteria. To prove that these organisms are present in the specimens, the characteristic colonies must be apparent in the cultures without the use of penicillin.

[7] Dienes, L., Proc. Soc. Exp. Biol. and Med., in press.

15734 P

Isolation of Pleuropneumonia-like Organisms from *H. influenzae* with the Aid of Penicillin.*

L. Dienes.

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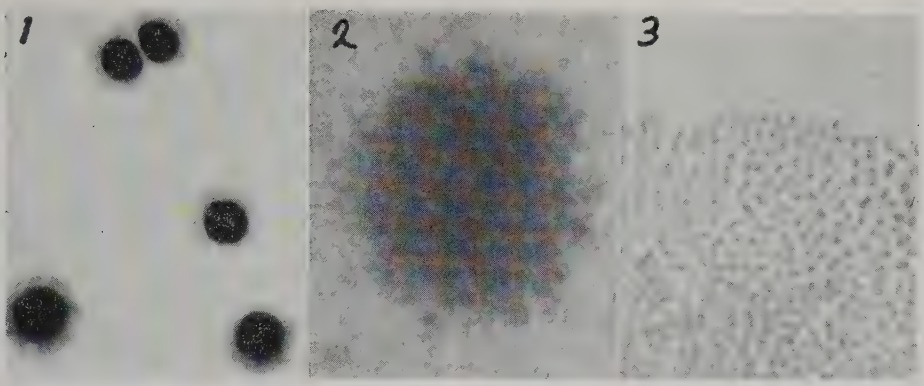
It has been previously noted[1] that certain strains of *H. influenzae* show a pleomorphism similar to that observed in *Streptobacillus moniliformis*[1] and bacteroides.[2] The bacilli swell up into large round bodies which either disintegrate into bacteria of usual appear-

* The expenses of this investigation have been defrayed in part by a grant from the Commonwealth Fund.

This is publication No. 92 of the Robert W. Lovett Memorial Foundation for the study of crippling disease.

[1] Dienes. L., *J. Bacteriology*, 1942, **44**, 37.

[2] Dienes, L., and Smith, W. E., *J. Bacteriology*, 1944, **48**, 125.



Photograph 1 shows well-developed colonies of the P.P.L.O. isolated from *H. influenzae* (× 90).

Photograph 2 shows a medium-sized colony with high magnification (× 3000). The colony is embedded in the agar and the shape of the individual organisms is not clearly defined.

Photograph 3 shows the edge of the colony of the parent *H. influenzae* strain (× 3000). The organisms are larger than in Photograph 2 and have a sharp contour.

Photographs 1 and 2 were made from stained agar preparations; No. 3 was made from an impression preparation after agar fixation.

ance or develop into tiny colonies resembling the organisms of bovine pleuropneumonia. The pleuropneumonia-like (P.P.L.) growth of *H. influenzae* remained very small, and it could not be determined whether or not they were able to grow indefinitely since they were always overgrown by regular bacterial forms.

Pierce[3] has observed that penicillin facilitates the isolation of L_1 from *Streptobacillus m.* by the elimination of the regular bacterial forms. After some experimentation with penicillin a procedure was found by which pleuropneumonia-like organisms (P.P.L.O.) can be isolated from cultures of *H. influenzae* and grown in pure culture.

H. influenzae is planted heavily on blood agar plates. One or several small troughs are made on the agar without cutting through it, and in each is placed a drop of solution containing 2000 units of penicillin per ml. After absorption of the penicillin solution the plates are made anaerobic by growing *B. prodigiosus* together with the culture and sealing the petri dish with paraffin. The plates are incubated at 30°C and 36°C and are opened after 1, 2, 3, and 4 days. Bacterial growth is completely inhibited in an area 2 to 3 cm in diameter around the trough. It is apparent in stained agar preparations that in the area of inhibition the originally deposited bacteria swell up into large round bodies sometimes as large as 15 to 20 micra without any indication of multiplication. In the border area where growth becomes apparent the organisms are swollen in a similar way. Further out they are replaced first by filaments and then by bacilli of the usual appearance. The swollen organisms do not show any sign of development if they are transplanted. However P.P.L. growth begins to develop from a few large bodies in the area in which bacterial growth is inhibited. Often after 48 hours many P.P.L. colonies are visible in stained agar preparations, and after 3-4 days a crop of tiny colonies, visible with the hand lens, grows in the zone of inhibition. These colonies are embedded in the agar and correspond in appearance and morphology to the organisms of the pleuropneumonia group.

The tiny colonies can be transplanted by cutting out a piece of agar containing them and smearing it on blood agar and boiled blood ascitic agar plates. The plates are treated with penicillin as were the original ones and are incubated anaerobically with *B. prodigiosus*. The cultures later can be grown

[3] Pierce, C. H., personal communications.

[4] Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 470.

without penicillin.

The culture so obtained differs from the parent organism in the following characteristics: (1) The organisms in young cultures are much smaller. They do not have a sharp contour like usual bacilli and they are very soft. The slightest injury deforms or destroys them. (2) The colonies do not grow on the surface of the agar but extend below the surface. The well-developed colonies as visible in Fig. 1 have the appearance typical of the pleuropneumonia group. (3) In older colonies the organisms swell into round bodies. (4) The organisms do not show satellite growth and are very resistant to penicillin. In appearance, staining and physical properties these organisms are very similar to the L_1 isolated from *Streptobacillus moniliformis,* to the corresponding organism isolated from bacteroides and to the whole pleuropneumonia group.

Five strains of *H. influenzae* were studied by the technic described. The strain from which the P.P.L.O. were most easily isolated was cultured from the sputum of a case of bronchiectasis treated with penicillin aerosol. The second strain, an *H. influenzae* Type B, from which large colonies of P.P.L.O. developed but which could not be kept in continuous cultivation was isolated from a blood stream infection. Immediately after isolation this culture was pleomorphic and produced tiny P.P.L. colonies. This property had been lost by the time experiments with penicillin were started. The other strains were not pleomorphic. Two, isolated from blood and from the conjunctiva respectively, produced a few fairly large P.P.L. colonies in the area of inhibition. The third strain, isolated from a sputum did not produce P.P.L. colonies. The strains with the exception of the second were studied immediately after isolation. Most freshly isolated strains of *H. influenzae* apparently can be induced to produce P.P.L.O. by exposure to penicillin. There is considerable variability among the strains concerning the ease with which P.P.L.O. are produced and with which they can be kept in cultivation. A similar variability has been observed in the viability of P.P.L.O. isolated from different bacteroides strains.[2] Use of the same technic has thus far not been effective in isolating P.P.L.O. from gonococci, *E. coli* and several strains of streptococci.

The role of penicillin in the growth of P.P.L.O. is unknown. It is possible that it consists mainly in the elimination of regular bacterial forms. The fact that swelling of bacteria induced by penicillin is very similar to the swelling occurring in naturally pleomorphic strains, may indicate that the effect of penicillin is more complex. The growth of P.P.L.O. does not seem to be a degenerative process because a viable organism, closely similar to an important group of pathogens, is produced.

Summary. With the aid of penicillin a strain of pleuropneumonia-like organism has been isolated from a culture of *H. influenzae.* This strain could be kept in continuous cultivation. Colonies of similar organisms were seen in 3 other cultures of *H. influenzae,* but their propagation in pure culture was not successful.

15735

Infectivity of *Trypanosoma cruzi* after Cultivation for Thirteen Years *in vitro* Without Animal Passage.

A. Packchanian and H. H. Sweets, Jr.

From the School of Medicine, University of Texas, Galveston, Texas.

It is an established fact that when *Trypanosoma lewisi* is cultured on N.N. (Novy and MacNeal) medium[1] and subcultured at 3- or 4-week intervals the strain remains infective to rats during the first year of cultivation. However, at the end of 2 years' *in vitro* cultivation the strain loses its ability to infect rats, even if a massive inoculum prepared from several cultures is injected into young or splenectomized rats.[2-4]

The question whether or not *Trypanosoma cruzi* also becomes attenuated after a similar period of *in vitro* cultivation had not been investigated previous to this study. Since we have in our possession a strain of *Tr. cruzi* which has been kept *in vitro* for nearly 13 years without animal passage, this problem was studied.

Methods and Materials. A naturally infected *Triatoma geniculatus* received from Panama was crushed and the intestinal contents of the insect were inoculated into a guinea pig. On June 20, 1932, a strain of *Tr. cruzi* was cultured from the guinea pig's blood on N.N. medium.[4] This culture has been maintained on N.N. medium at room temperature or at 25°C. Subcultures were made usually every 4 to 8 weeks; occasionally a longer interval was allowed to elapse before transfers were made.[5] During this 13-year period, the strain was subcultured 81 times without any animal passage.

The 81st subculture of this strain of *Tr. cruzi* was inoculated to 8 mice *(Mus musculus)*. The blood of these animals was examined microscopically at various intervals following inoculation, devoting about 5 minutes to each preparation. Xenodiagnosis (which consists of feeding normal *Triatoma* on the animals and subsequently examining the insect for infection with flagellates), was used on 2 mice. At varying times after inoculation, the mice were sacrificed and the blood was inoculated to N.N. medium. The tubes were covered with rubber caps, incubated at 25°C and examined after about 3 weeks for motile flagellates.

Results. The trypanosomes were demonstrated by direct microscopic examination in 4 out of 8 mice from 27 to 48 days after inoculation.

One of the xenodiagnostic tests was positive 50 days after injection, while the other was found negative 46 days following inoculation.

The mice were killed from 34 to 53 days after inoculation, and the blood of all 8 animals gave positive cultures for flagellates.

Summary. A strain of *Trypanosoma cruzi* cultured from experimentally-infected guinea pig on N.N. medium was subcultured at various intervals on N.N. medium for 13 years without animal passage. At the end of this period the strain was found to be still capable of producing infection in experimental animals.

The presence of *Tr. cruzi* was microscopically demonstrated in the circulating blood of 50% of the inoculated animals. However, cultures made from the heart blood of all the animals on N.N. medium were positive for *Tr. cruzi*.

1 Novy, F. G., and MacNeal, W. J., *J. Infect. Dis.*, 1904, **1**, 1.

2 Novy, F. G., Perkins, W. A., and Chambers, R., *J. Infect. Dis.*, 1912, **11**, 411.

3 Behrens, C. A., *J. Infect. Dis.*, 1914, **15**, 24.

4 Packchanian, A., *Science*, 1934, **80**, 407.

5 Packchanian, A., *J. Parasitol.*, 1943, **29**, 275.

15736

Microscopic Historadiographic Technic for Locating and Quantitating Radioactive Elements in Tissues.

K. M. Endicott and Herman Yagoda. (Introduced by F. S. Daft.)

From the Pathology Laboratory and the Industrial Hygiene Research Laboratory, National Institute of Health, Bethesda, Md.

Accurate quantitative microscopic studies of tissue and cellular distribution of radioactive elements which decay with emission of alpha particles may be made quite easily by the technics to be described. The essential points in the technic are (1) the use of fine-grained silver-bromide emulsions with selective response to alpha radiation,[1,2] (2) the mounting of tissue sections permanently on the emulsion at the beginning of the exposure, (3) examination of such preparations at 400 diameters in dark-field illumination for determination of number of alpha particle tracks per unit area of emulsion, and (4) examination of such preparations at 1000-2000 diameters for determination of the precise cellular location of the radioelement by tracing straight tracks through the emulsion to their points of origin in the tissue.

The following technics have been evolved in the study of radium F (polonium) which decays with emission of alpha particles and very low intensity gamma rays. The polonium is administered in physiological saline buffered with $NaHCO_3$. Tissues are fixed in buffered neutral 4% aqueous HCHO for 48 hours. Bones are decalcified in 5% aqueous HCOOH. Tissue blocks are dehydrated in acetone, cleared in gasoline, embedded in paraffin in a vacuum oven and sectioned at 5 μ. The ribbons are floated off cold water onto the photographic emulsion (Eastman alpha particle plate No. 329,489). All manipulations of the undeveloped photographic plates are carried out in a room illuminated by the appropriate safelight. The preparation is dried quickly in a current of air and is placed in a light-tight container for the remainder of the exposure period. At the end of the exposure period the paraffin is removed by placing the plate in xylene for 5 minutes and the plate is dried in a current of air. The plate is developed in D-19, hardened in SB-4, and fixed in F-5 (Eastman Kodak formulae) at room temperature after which it is washed in filtered tap water for 2 hours. The plate may be stained immediately or dried and stored for staining later. The plate is stained in freshly prepared Weigert's acid-iron hematoxylin for 2 minutes, washed in tap water 5 minutes, dehydrated in 3 changes of acetone, cleared in 1:1 acetone-xylene and in 3 changes of xylene. A drop of xylene-clarite is placed on the tissue and the preparation is covered with a glass coverslip.

Crude quantitation of the relative distribution of polonium in tissues may be obtained by microdensitometer analysis of the developed emulsion. A much more precise local analysis is obtained by counting the number of particle tracks per unit area of emulsion. The amount of polonium present is directly proportional to the number of tracks when the experimental conditions are constant. Determination of relative distribution is thus reduced to comparison of number of tracks. The actual content of polonium per unit of tissue can be calculated from decay constant, length of exposure, spatial relationship of emulsion and tissue, volume of tissue, etc., but this calculation is not ordinarily necessary.

In addition to the quantitative aspects, there is another feature of great interest to the biologist. This is at present limited to the alpha ray emitting elements. In preparations in which the tissue is mounted permanently on the emulsion at the beginning of the exposure, one can locate the point of origin of the alpha particle with great precision. This depends upon the extremely shallow depth of focus at high magnifications (500-2000×). By turning the fine ad-

1 Yagoda, H., *Am. Mineralogist*, 1946, **31**, 87.

2 Yagoda, H., *Am. Mineralogist*, 1946, **31**, 462.

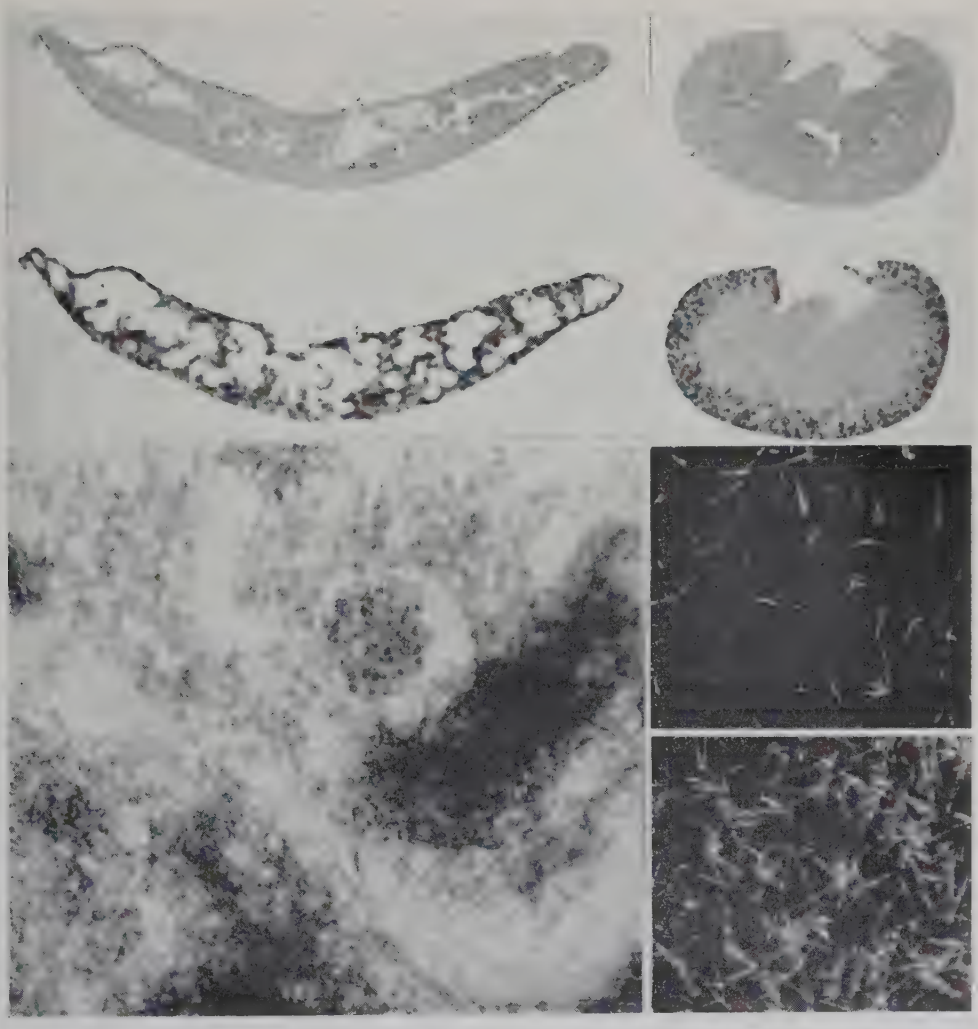


PLATE I.

1. Photomicrograph of mouse spleen containing polonium, hematoxylin, and eosin. × 5.
2. Temporary contact historadiograph prepared from (1). × 5.
3. Photomicrograph of mouse kidney containing polonium.
4. Temporary contact historadiograph prepared from (3).
5 and 6. Dark field views of alpha particle tracks from an area of low and medium polonium content (salivary gland). × 400.
7. Photomicrograph of permanent tissue-photographic emulsion preparation of mouse kidney containing polonium showing renal cortex with extremely dense areas of alpha particle tracks beneath distal convoluated tubules. ×500.

justment knob of the microscope, one can trace each alpha particle track from its end to its beginning and thus to the precise point in the tissue from which it originated. It is thus possible to determine whether the parent atom was located in cytoplasm or nucleus of a given cell. The cellular distribution of radioelements which decay solely with emission of beta or gamma radiations probably cannot be studied by such methods.

Macro images for low power photomicrography are best prepared by one of two technics in which the tissue is not mounted on the emulsion permanently. The tissue ribbon may be mounted on a glass slide, deparaffinized in xylene, coated with celloidin, and placed in contact with the photographic emulsion. At the end of the exposure the glass slide and the photographic plate are separated, the plate being developed and the

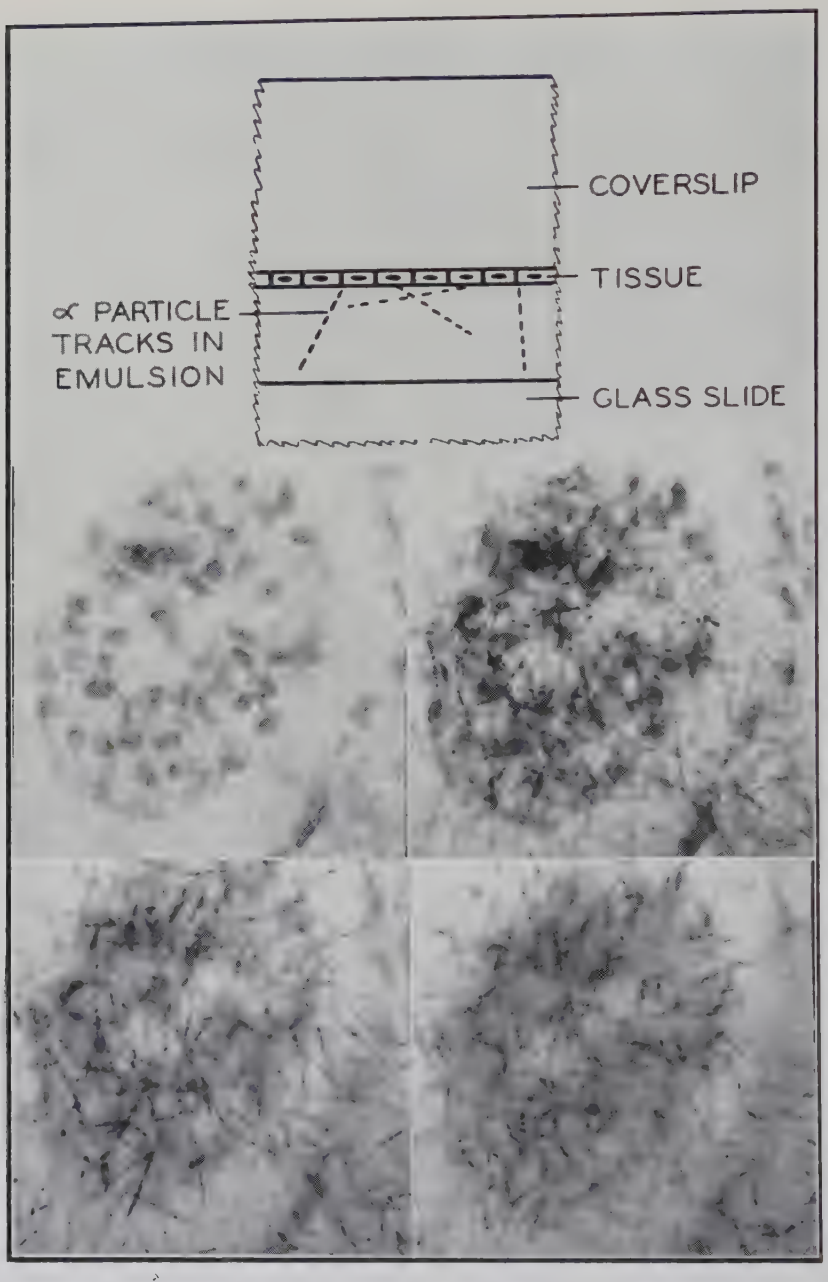


PLATE II.

1. Schematic drawing of permanent tissue-photographic emulsion preparation.

2, 3, 4, 5. Photomicrographs of renal glomerular tuft and underlying photographic emulsion at successively deeper focus. Permanent tissue-photographic emulsion preparation. Hematoxylin and eosin stain. × 1000.

tissue slide being stained by any one of the many ordinary methods after removing the celloidin with acetone. Equally satisfactory and in many cases superior historadiographs are obtained if the plane-surfaced paraffin block, which remains after the sections are cut, is placed in contact with the photographic plate for preparation of the historadiograph. The image is compared with the stained section last cut from the block.

15737 P

Infection of Mice with Tubercle Bacilli Grown in Tween-Albumin Liquid Medium.

Cynthia Pierce, Rene J. Dubos, and Gardner Middlebrook.

From the Laboratories of the Rockefeller Institute for Medical Research, New York City.

Tubercle bacilli, growing diffusely in liquid media containing a water dispersible ester of oleic acid (Tween 80), retain unaltered many of their morphological and biological properties.[1,2] The present paper describes the virulence of these cultures for mice of different genetic backgrounds inoculated under various experimental conditions. The cultures used in the infection tests to be reported were grown for 7-10 days in a medium containing 0.05% Tween 80 and 0.2% bovine albumin (serum Fraction V).[2] Macroscopically, these cultures appear homogeneous, but in reality consist of microscopical clumps. Their density corresponds to approximately 0.20 mg/cc in terms of dry weight of bacilli.

Mice, 3 to 6 weeks old, of the Rockefeller Institute strain inoculated intravenously with 0.01 cc of whole culture begin to lose weight during the second week after infection and die in 3 to 4 weeks with a disease primarily pulmonary. The pulmonary lesions consist of discrete and confluent nodules varying in size, pearly gray in color and firm in consistency. Stained microscopic sections show innumerable tubercle bacilli in these areas. Heart muscle is often infiltrated with very many small lesions. When the inoculum is reduced 10-fold to 0.001 cc, only an occasional animal dies after the 4th week of infection, although all animals sacrificed at this time show pulmonary lesions.

Much larger amounts of culture (0.5-1.0 cc) are required to produce death within 3 to 4 weeks when the infective dose is introduced by the intraperitoneal route. This minimal lethal dose can be reduced appreciably by adding fresh egg yolk to the bacterial suspension.

Fresh egg yolk is diluted with an equal part of 0.85% saline. One volume of culture is emulsified with one volume of the diluted egg yolk suspension immediately prior to infection. The data presented in Table I illustrate the enhancement of infection by the addition of fresh egg yolk to the culture.

The infection tests just described were carried out with the classical H37Rv culture obtained through the courtesy of Mr. William Steenken of the Trudeau laboratory. Similar results have been obtained with other human and bovine cultures of tubercle bacilli recently isolated from pathological materials. On the other hand, saprophytic acid-fast bacilli and variants derived from pathogenic strains but known to be devoid of pathogenicity for guinea pigs and chick embryos, fail to establish a progressive disease in mice even when injected in large amounts. Table II illustrates the results of inoculating mice (Rockefeller Institute strain) by the intravenous route with 2 different variants of the H37 culture of human tubercle bacilli, H37 (virulent) and H37Ra (avirulent). Even with the addition of egg yolk, 0.2 cc of H37Ra culture fails to establish a progressive infection or to produce grossly visible lesions.

Fifteen different strains of mice have been compared for susceptibility to tuberculous infection by the intravenous and intraperitoneal routes. The progress of the disease was determined by weight changes of the animals at weekly intervals, length of survival, numbe and extent of macroscopic pulmonary lesions, and enlargement of the spleen. Whatever the mode of infection, and whatever the criteria used for evaluating the severity of the disease, the differences observed are consistent and permit the recognition of marked

1 Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

2 Dubos, R. J., Davis, B. D., Middlebrook, G., and Pierce, C., *Am. Rev. Tuberc.*, 1946, **54**, 204.

TABLE I.
Effect of Egg Yolk upon Infection of Mice with Tubercle Bacilli (H37) via Intraperitoneal Route.

Culture H37	Egg yolk suspension	Wt. at weekly intervals after infection				No. dead/ Total inoculated
		Initial	1 wk	2 wk	3 wk	
cc	cc	g	g	g	g	
0.25	0	20.0	20.2	22.6	24.2	0/6
0.25	0.25	20.5	20.7	18.0	Dead	6/6

TABLE II.
Comparison of H37 (Virulent) and H37Ra (Avirulent) Strains Inoculated Via Intravenous Route.

Culture	Egg yolk suspension		Wt at weekly intervals after inoculation					No. dead/ Total inoculated
			Initial	1 wk	2 wk	3 wk	4 wk	
	cc	cc	g	g	g	g	g	
H37	0.01	0	18.3	21.3	22.2	19.7	19.2	5/6
H37Ra	0.2	0	18.3	21.6	24.2	25.9	26.7	0/6
H37Ra	0.2	0.1	18.3	20.9	23.3	24.6	25.3	0/6

differences in susceptibility among the different strains of mice. In general, albino mice derived from the so-called Swiss strain are somewhat more resistant than the white mice of the Rockefeller Institute strain. On the contrary, a number of other strains such as C57 black, dba, and wild mice *(Mus musculis domesticus)* are markedly more susceptible. The C_3H strain is intermediate between the latter group and the Rockefeller Institute strain.

Albino Swiss mice die within 3 weeks following intravenous injection of 0.05 cc of H37 culture; they survive and exhibit only limited pulmonary lesions when infected with smaller doses. On the other hand, 0.003 cc of culture is sufficient to cause death of dba mice within 3 weeks; moreover, animals of this strain, sacrificed 4-5 weeks after intravenous injection of 0.00003 (corresponding to 0.000005 mg dry weight becilli), show extensive pulmonary lesions often involving whole lobes.

15738

Alpha Naphthylthiourea (ANTU) in Dogs: Electrophoretic and Cholesterol Studies on Blood Plasma and Pleural Effusion.*

ALFRED CHANUTIN, E. C. GJESSING, AND STEPHAN LUDEWIG. (Introduced by C. P. Richter.)

From the Biochemical Laboratory, University of Virginia, Charlottesville.

Richter[1] recently described a new rat poison, alpha naphthylthiourea (ANTU), which exerts its toxic effect within a few hours by increasing the permeability of the pulmonary and pleural capillaries. In the dog and rat, the pulmonary edema and pleural effusion cause death. An analysis of the electrophoretic patterns of the serum and effusion fluid in animals poisoned with ANTU provides an approach to the problem of diffusion of proteins across damaged capillary walls.

* This work was done under contract with the Medical Division of the Chemical Warfare Service.

[1] Richter, C. P., *J. Am. Med. Assn.*, 1945, **129**, 927.

TABLE I.
Analyses of the Electrophoretic Patterns of Serum and Pleural Effusion After Lethal Doses of ANTU.

Hr after ingestion	% area				Mg N %			
	Albumin	α_1 α_2	α_3 β_1	β_2 γ	Albumin	α_1 α_2	α_3 β_1	β_2 γ
75 mg/kg ANTU in gelatin capsule by mouth.								
Dog 1: Wt—27.3 kg.								
Serum.								
Control	51	14	35		492	135	338	
4	49	17	34		480	166	333	
6	48	16	36		446	149	335	
10	48	21	32		385	168	257	
12	45	24	32		373	199	265	
13½	47	18	35		307	118	229	
15	43	22	34		279	143	221	
Pleural Effusion.								
15	54	12	34		388	86	244	
Dog 2: Wt—20.6 kg.								
Serum.								
Control	47	16	30	5	540	184	345	58
7	46	12	34	6	520	136	384	68
14	45	13	35	6	476	138	371	64
15	46	15	33	6	527	172	378	69
16	47	13	34	7	446	124	323	67
17	52	10	31	7	505	97	300	68
Pleural Effusion.								
15	46	12	36	5	324	85	254	35
16	47	15	38		329	105	266	
17	53	10	37		366	69	255	
Dog 3: Wt—20 kg.								
700 mg ANTU intravenously injected in a 6% acacia-saline mixture.								
Hr after injection			Serum.					
0	40	19	33	8	440	209	363	88
6	40	18	33	9	388	175	320	87
7	41	18	35	6	400	176	341	58
7½	37	23	31	9	396	246	332	96
Pleural Effusion.								
6	49	13	38		318	85	247	
7½	49	10	34	7	360	74	250	52
Dog 4: Wt—15.9 kg.								
560 mg ANTU injected intravenously.								
Serum.								
0	47	14	32	7	545	162	371	81
7	39	19	33	9	392	177	307	84
Pleural Effusion.								
7	53	12	28	7	379	86	200	50

Richter also observed that if a rat ingests a sublethal dose of ANTU, its tolerance for larger amounts develops rapidly and persists for a comparatively long period. The thiourea portion of the ANTU molecule probably affects the thyroid metabolism of these animals. A number of investigators have found that thiourea and its derivatives depress the functional activity of the thyroid gland by interfering with the synthesis of thyroxine and diiodotyrosine.[2,3] In man, thiourea administration causes a rise of blood cholesterol[4] which may be expected in hypothyroidism. Moore, Levin, and Smelser[5] showed than an α-globulin component of rat sera increases after thyroidectomy or after thiouracil feeding.

This report deals with (a) the protein and cholesterol concentrations of the serum and pleural effusion in dogs dying after oral or intravenous administration of ANTU; and

2 Astwood, E. B., *J. Am. Med. Assn.*, 1943, **122**, 78.

3 Chen, K. K., *Ann. Rev. Physiol.*, 1945, **7**, 677.

4 Jennings, L. M., Mawson, C. A., and Tindall, W. J., *Lancet*, 1944, **2**, 91.

5 Moore, D. H., Levin, L., and Smelser, G. K., *J. Biol. Chem.*, 1945, **157**, 723.

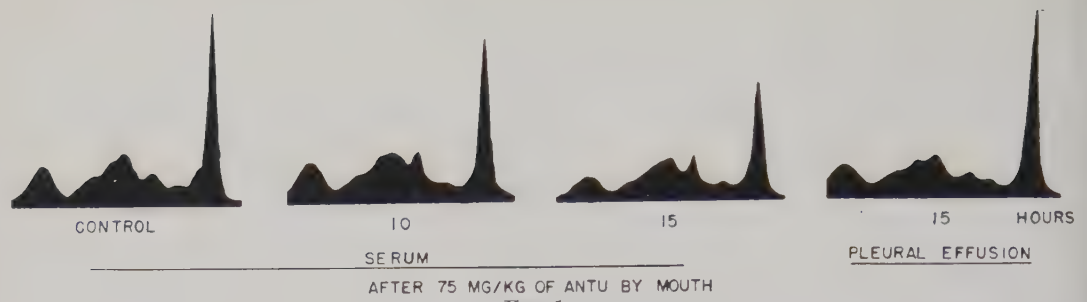


FIG. 1.

Electrophoretic patterns for the serum and pleural effusion of Dog 1 after the administration of ANTU.

(b) the protein and cholesterol concentrations of the serum of dogs which had developed a tolerance to large amounts of this drug.

Methods. Electrophoretic analyses were done in the Tiselius apparatus, using the scanning method of Longsworth[6] and single section cell of 11.0 ml capacity. The serum was diluted 1.5 times with barbiturate buffer, ionic strength 0.1, at pH 8.6. Electrophoresis proceeded for 120 minutes at 15 m. amp. and 200 volts, with a potential gradient of 6.0 volt per cm at 2.0°C.

Cholesterol was determined on plasma by the Schoenheimer-Sperry procedure[7] with the aid of an Evelyn photoelectric colorimeter with filter No. 660; known amounts of recrystallized cholesterol were used for calibration. The micro-Kjeldahl method was used for total and nonprotein nitrogen.

Results. Protein Distribution. The electrophoretic analyses of 4 dogs given ANTU orally and intravenously are presented in Table I. Owing to the indistinct nature of the electrophoretic patterns of these sera, the proteins are grouped as (1) albumin and α_1, (2) $\alpha_2 + \alpha_3$, (3) $\beta_1 + \beta_2$, and (4) γ globulins, or a combination of (3) and (4). In group (1), α_1 globulin represents a small proportion of the protein. The data are expressed as percent of total protein and as milligrams percent of nitrogen.

Acute Poisoning. Satisfactory sampling of blood was attained in 2 of 6 dogs which were given ANTU in gelatin capsules by mouth, and 2 of 4 dogs intravenously injected with ANTU.[8] In all animals the pulmonary edema was very marked and responsible for their death. Varying amounts of blood-tinged pleural effusion were seen in 9 of the 10 dogs. Usually some respiratory distress was exhibited several hours before death, and great difficulty during the last hour. It was impossible to obtain sufficient serum (10-15 ml) from 6 dogs despite the fact that 100 to 200 ml of blood were obtained when the animal was sacrificed by exsanguination. The blood, of tarry consistency when freshly drawn, was allowed to stand for several hours and was then centrifuged at high speeds. The clot was not firm and did not retract over a period of at least 5 hours. Standing for longer periods yielded a serum which was markedly hemolyzed.

The essential details concerning the 4 satisfactory experimental animals are discussed.

Dog 1. (Table I). Blood was drawn at intervals and the dog sacrificed 15 hours after oral administration of ANTU. The serum albumin concentration decreased markedly and the nitrogen concentration of the $\beta_1 + \beta_2 + \gamma$ globulin group was also lowered.

In the effusion fluid, the albumin concentration was greater than that of the serum at the time of death; the $\alpha_2 + \alpha_3$ group was smaller. The electrophoretic patterns of serum and pleural effusion (Fig. 1) show a striking similarity.

Dog 2. (Table I). The chest was tapped at intervals and the first sample of effusion

[6] Longsworth, L. G., *J. Am. Chem. Soc.*, 1939, **61**, 529.

[7] Schonheimer, R., and Sperry, W. M., *J. Biol. Chem.*, 1934, **106**, 745.

[8] Drinker, C. K., *Pulmonary Edema and Inflammation*, Harvard University Press, 1945, 39-43.

TABLE II.
Electrophoretic Analyses of Sera of Dogs 5 and 6 After Multiple Oral Doses of ANTU.
Grams Protein in 100 ml Serum.

Dog No.	Experimental day	ANTU mg/kg	% distribution			Mg N in 100 ml serum		
			Alb.	$\alpha_1 + \alpha_2$	$\beta + \gamma$	Alb.	$\alpha_1 + \alpha_2$	$\beta + \gamma$
5	1	10	51	14	35	465	130	326
	3	10						
	4		47	18	35	454	174	331
	6	15						
	8	25	46	18	36	457	172	352
	10	40	46	21	33	444	206	321
	13	60	42	22	36	411	219	358
	15	75	40	24	36	387	235	355
	17	75	42	23	35	400	214	337
	20	150	39	22	39	390	224	392
	22		48	22	30	515	232	323
	27		46	22	32	470	230	331
	31		44	21	35	403	198	318
6	1	10	56	16	28	385	110	195
	3	10						
	4		49	25	26	385	198	201
	6	15						
	8	25	48	20	32	395	164	257
	10	40	47	21	32	380	168	257
	13	60	49	20	31	406	161	254
	15	75	45	21	34	374	174	278
	17	75	47	21	32	376	172	249
	20	150	48	19	32	416	164	270
	24		49	20	31	388	155	240
	27		50	24	26	427	208	217
	41		50	21	29	424	180	249

fluid obtained 15 hours after oral administration. The percentage composition of the serum proteins remained constant throughout the experiment, but the protein concentrations decreased. Appreciable decreases in the milligrams percent of nitrogen occurred in all groups except the γ globulin.

The percentage distribution of the protein groups was almost identical for the serum and effusion fluid at the respective periods. The total nitrogen concentration of the effusion was less than that of the serum, and therefore the content of the various protein groups was proportionately smaller.

Dog 3. (Table I). ANTU was injected intravenously and the chest was tapped hourly. The first effusion fluid was obtained 6 hours after injection at which time blood was also drawn. The dog showed signs of distress shortly afterwards and died 7½ hours after injection. Respiration ceased before the animal could be exsanguinated. Blood was drawn from the heart and approximately 200 ml of effusion fluid were obtained.

The changes in the concentration of the serum fractions were not pronounced. The percentage of the albumin fraction in the pleural effusion was greater than in the serum and the milligram percentage of nitrogen was slightly lower. The nitrogen concentrations of the globulin groups were much lower than those of the serum.

Dog 4. (Table I). This animal was exsanguinated 7 hours after intravenous injection of ANTU. The serum albumin concentration was decreased markedly at death and the globulin groups were only slightly affected. The albumin nitrogen concentration of the effusion fluid was about the same and the concentrations for the globulins were less than those of the serum.

Chronic Poisoning. Two dogs, fed increasingly larger amounts of ANTU in a gelatin capsule, developed a tolerance for doses ordinarily lethal to control animals. The perti-

TABLE III.
Plasma and Pleural Effusion Cholesterol.
Dog given 75 mg/kg of ANTU by mouth.

Hr after ANTU	Total cholesterol, mg %
Plasma.	
Control	95
4	97
6	107
10	91
12	87
13½	79
15	72
Pleural Effusion.	
15	67

nent data for drug administration and for protein distribution in the serum are shown in Table II. There is no typical change in the protein distribution, except a moderate increase in α globulin concentration which can be correlated with the development of tolerance for ANTU.

Plasma Cholesterol. The data for the plasma and effusion cholesterol concentrations after acute poisoning (Table III) indicate a definite decrease in the plasma a few hours before death. The cholesterol concentrations of the plasma and pleural effusion were the same.

Chronic poisoning in 2 dogs was accompanied by a marked increase in plasma cholesterol concentration which probably reflected the "chemical thyroidectomy" caused by ANTU (Table IV). An immediate increase in cholesterol was seen after the first small doses. The values continued to increase while the ANTU was administered. After its discontinuance, the plasma cholesterol concentration decreased rapidly.

TABLE IV.
Effect of Chronic ANTU Feeding on Plasma Cholesterol of Dogs.

Day of exper.	ANTU administered mg/kg	Plasma cholesterol mg %	
		Dog 5	Dog 6
1	10	95	118
3	10		
4		153	165
6	15		
8	25	166	176
10	40	194	195
13	60	235	191
15	75	255	215
17	75	287	228
20	150	254	235
22		215	235
24		216	178
27		150	127
31		135	111
41		98	87

Discussion. In ANTU poisoning, the decrease in the concentration of the protein fractions, particularly in albumin, is apparently a result of the loss of plasma into the lungs and effusion. In the 2 experiments in which the proteins of the pleural effusion were measured before and at death, the albumin concentration increased with time; the data for the globulins are insufficient for drawing any conclusion.

Results of a study of plasma and effusion proteins in a variety of diseases by Luetscher[9] indicate that the "albumin is present in the same or higher proportion in the effusion, as compared with plasma." Cohn[10] believes that the factor determining diffusion of a protein across a permeable membrane "should be an inverse function of the length of its molecule." This idea is borne out by the data presented by Luetscher,[9] and by the present experiments.

The effect of ANTU on cholesterol metabolism is striking. The increases noted in chronic experiments are probably associated with the thiourea portion of the molecule and its action on the thyroid. Direct observations on the thyroids were not made.

Summary and Conclusion. Oral and intravenous administration of lethal doses of alpha naphthylthiourea (ANTU) to 4 dogs caused death by affecting the permeability of pulmonary and pleural capillaries, which resulted in marked pulmonary edema and pleural effusion. Electrophoretic and nitrogen determinations of the sera showed consistent decreases in the concentration of albumin several hours before death. The proteins of the pleural effusion showed a higher albumin : globulin ratio than those of the serum. The total plasma cholesterol concentration decreased shortly before death and

[9] Luetscher, J. A., Jr., *J. Clin. Invest.*, **1941**, **20**, 99.

[10] Cohn, E. J., *Proc. Am. Philosoph. Soc.*, **1944**, **88**, 159.

was approximately the same in the effusion.

Dogs fed increasingly greater amounts of ANTU during frequent intervals develop a tolerance for large doses of the drug. An increase in the serum α globulin concentration appeared to be a characteristic finding in the development of the tolerance. The plasma cholesterol concentration increased markedly during drug administration and diminished when it was discontinued.

15739 P

Folic Acid (Pteroylglutamic Acid)* Studies: Hematologic Remissions in Pernicious Anemia.

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Since the *Lactobacillus casei* factor (folic acid) was synthesized in 1945 by Angier and others,[1] many clinical investigators have clearly demonstrated its effectiveness in the treatment of Addisonian pernicious anemia, the anemia of sprue, nutritional macrocytic anemias and the macrocytic anemias of pregnancy. It has likewise been shown that folic acid is quite effective in producing a complete hematologic remission in certain severe macrocytic hyperchromic anemias of infants which are characterized by poor nutrition, a transient histamine fast achlorhydria and a marked megaloblastic hyperplasia of the bone marrow. The treatment of certain human macrocytic anemias with folic acid (pteroylglutamic acid) has been reported by Darby, Jones and Johnson,[2] Spies and co-workers,[3] Moore and co-workers,[4] Goldsmith,[5] Doan, Wilson and Wright,[6] Zuelzer[7] and others. These extensive studies have been made possible by the chemical identification and synthetic preparation of folic acid (pteroylglutamic acid) in sufficient amounts to permit extensive clinical investigation in several fields. Within the past 12 months we have used synthetic folic acid in treating a great number of different blood dyscrasias. The full report of our investigations will be published in a later communication.

This report deals with the effectiveness of folic acid in the treatment of 4 untreated cases of classical Addisonian pernicious anemia in severe relapse. All of the patients in this group were hospitalized for thorough study and control observations prior to the institution of folic acid therapy. In addition to the usual laboratory procedures for classifying and studying an anemia, sternal marrow aspirations, complete X-ray and fluoroscopic studies of the gastrointestinal tract and gastric analyses were done on each patient. None of these patients exhibited involvement of the central nervous system. A typical hematologic remission is graphically illustrated by the erythrocyte, hemoglobin and reticulocyte response indicated in Chart 1.

* The synthetic folic acid (Folvite) used in this clinical investigation was kindly supplied by Stanton M. Hardy, M.D., Medical Director, Lederle Laboratories, American Cyanamid Company, Pearl River, N.Y.

† The authors wish to express their gratitude to Miss Helen May Holt for her technical assistance in this investigation.

1 Angier, R. B., and others, *Science*, 1945, **102**, 227.

2 Darby, W. J., Jones, E., and Johnson, H. C., *J. A. M. A.*, 1946, **130**, 780.

3 Spies, T. D., Vilter, C. F., Koch, M. B., and Caldwell, M. H., *South. M. J.*, 1945, **38**, 707.

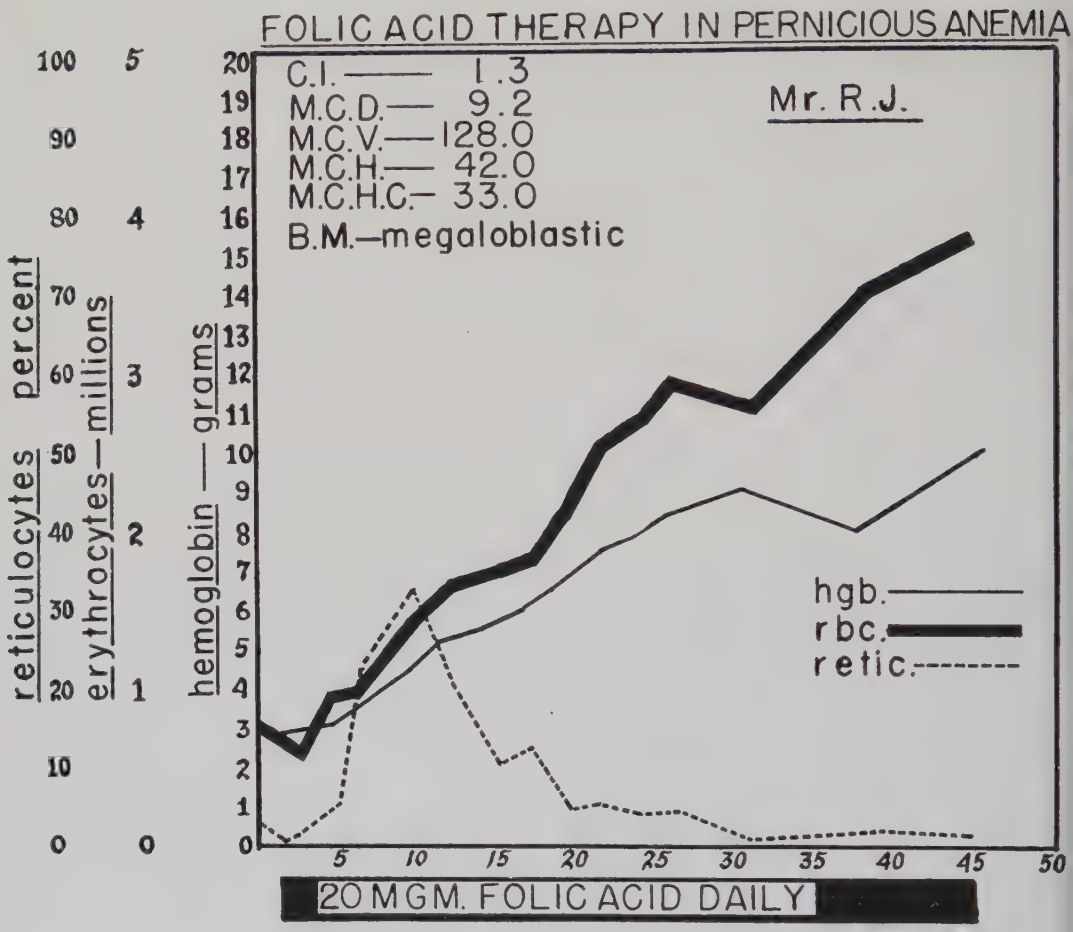
4 Moore, C. V., Bierbaum, O. S., Welch, A. D., and Wright, L. D., *J. Lab. and Clin. Med.*, 1945, **30**, 1056.

5 Goldsmith, G. A., *J. Lab. and Clin. Med.*, 1946, **31**, 1186.

6 Doan, C. A., Wilson, H. E., Jr., and Wright, C. S., *Ohio State M. J.*, 1946, **42**, 139.

7 Zuelzer, W. W., *J. A. M. A.*, 1946, **131**, 7.

CHART I



Similar responses were obtained in 3 other cases.

Case Mr. R. J. The initial erythrocyte count was 770,000 cu mm and hemoglobin was 3 g. This patient was given 20 mg of folic acid (pteroylglutamic acid) daily by mouth, and the hematologic response was prompt. At the present time this patient is in a state of complete hematologic remission (7 months later).

Discussion. Subjective and objective clinical improvement appeared to coincide with the time of maximum reticulocytosis. In all of these cases clinical improvement was rapid and equally as good as is ordinarily observed in pernicious anemia patients receiving adequate liver extract therapy. In the folic acid-treated group it appears that maximum reticulocytosis is greatest in those cases with erythrocyte levels below one million. Our group of patients has been maintained on 10 mg of folic acid daily by mouth.

It is clearly evident that folic acid (pteroylglutamic acid) will produce a satisfactory hematologic remission in cases of classical Addisonian pernicious anemia, and it appears that these patients can be maintained in remission on a daily oral dose of about 10 mg. None of our patients on this therapy have had or developed central

ervous system disease, so that complete valuation of folic acid in that respect can- ot be made at this time. However, Doan nd Moore[8] independently have recently re- orted the development and progression of entral nervous system disease in pernicious nemia patients while receiving folic acid nerapy. Folic acid appears to be equally s effective as liver extract therapy in the reatment of uncomplicated cases of Addi- onian pernicious anemia. While folic acid effective in producing a favorable hematologic remission in cases of pernicious anemia, it is certain that liver extracts contain active material other than this compound, since the potency of such extracts is greater than can be accounted for on the basis of their folic acid content.

Conclusion. 1. Folic acid (pteroylglutamic acid) is as effective as liver extract in correcting the hematologic deficiency in Addisonian pernicious anemia. 2. Further experience is needed before one can evaluate the effectiveness of this factor in preventing development and progression of central nervous system disease.

8 Doan, C. A., and Moore, C. V., personal communication to R. R. K.

15740

Effect of Frequency of Hypothalamic Stimulation upon Bladder Response.

BÖRJE UVNAS. (Introduced by H. W. Magoun.)

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Bladder contraction has been observed by many investigators as the result of the stimulation of the hypothalamus (Karplus and Kreidl,[1] Hess[2] and others). From their experiments on cats Kabat, Magoun and Ranson[3] and Magoun[4] concluded that a center for the contraction of the bladder is localized in a region just rostral to the hypothalamus and that fibres from this region run caudalward through the lateral part of the hypothalamus. Relaxation of the bladder was only infrequently observed but points giving inhibitory responses were found in different regions of the diencephalon (Kabat *et al.*[3]) Beattie and Kerr[5] claimed the existence of centers for the increase of bladder tonus in the anterior hypothalamus and for inhibition in the posterior hypothalamus and upper midbrain.

The blood pressure and respiratory responses to hypothalamic stimulation have been shown to be strongly influenced by the frequency of the stimulus (Hare and Geohegan,[6] Bronk, Pitts and Larrabee,[7] Berry, McKinley and Hodes[8]). In the present experiments the effect of frequency of hypothalamic stimulation upon bladder response was observed.

Methods. Twenty-four cats under intravenous chloralose anesthesia (50 mg per kg body weight) were used. The hypothalamus was stimulated by a bipolar electrode, made by cementing together 2 lengths of enameled nichrome wire, and oriented with the Horsley-Clarke technic. A thyratron stimulator (Rahm), delivering impulses within a frequency range of 3.5 to 1000 per second, was

1 Karplus, J. P., and Kreidl, A., *Arch. ges. Physiol.*, 1909, **129**, 138.

2 Hess, W. R., *Arch. f. Psychiatr.*, 1936, **104**, 548.

3 Kabat, H., Magoun, H. W., and Ranson, S. W., *J. Comp. Neurol.*, 1936, **63**, 211.

4 Magoun, H. W., *Am. J. Physiol.*, 1938, **122**, 30.

5 Beattie, J., and Kerr, A. S., *Brain*, 1936, **59**, 92.

6 Hare, K., and Geohegan, W. A., *J. Neurophysiol.*, 1941, **4**, 266.

7 Bronk, O., Pitts, R. F., and Larrabee, G., *Res. Publ. Nerv. Ment. Dis.*, 1940, **20**, 323.

8 Berry, C., McKinley, W., and Hodes, R., *Am. J. Physiol.*, 1942, **135**, 338.

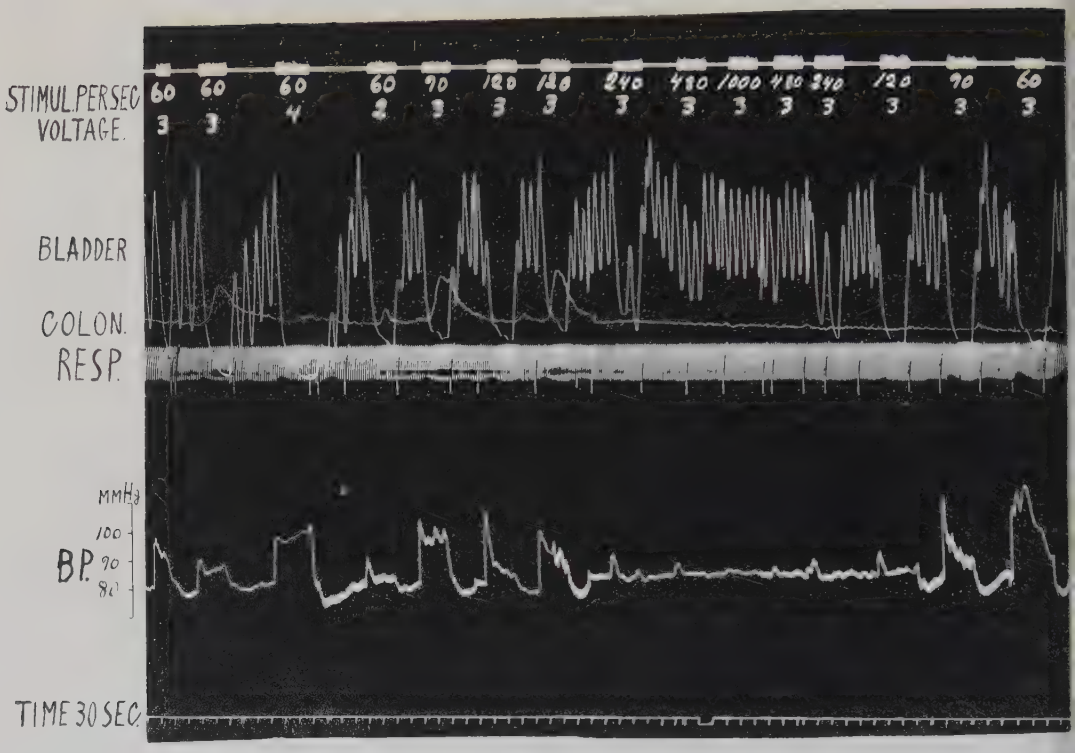

FIG. 1.
Alterations in bladder, colon, blood pressure, and respiration on stimulation of a point in the posterior part of the supraoptic region. Frequency varied.

employed. The pressure of the bladder was recorded by a water manometer connected to a cannula inserted into the bladder through the urethra. In about half the cases the brain was prepared for microscopical examination.

Results. Stimulation of numerous points in the hypothalamus and the preoptic region yielded changes in the intravesical pressure and the peristaltic activity of the bladder. The effects could be either excitatory or inhibitory, the response obtained being strongly influenced by the frequency of the stimulus used. It was an almost regular finding that high frequency stimuli (60/sec. or more), if effective, yielded inhibitory and low frequency stimuli (3.5/sec.), if effective, excitatory responses. The ranges of frequency usually giving inhibitory or excitatory responses are shown in Fig. 1 and 2. As seen, stimuli of a frequency from 15 to 240/sec. yielded a significant inhibition of the bladder. The intravesical pressure as well as the peristaltic activity was depressed. The frequency range giving a pronounced fall of the intravesical pressure and a complete inhibition of the peristalsis in this experiment was observed to be between 30 and 120/sec. At 15/sec. a small initial rise was observed to precede the fall of intravesical pressure and the pressure started to rise before the end of the stimulation. A similar tendency to "escape" was seen at the stimulation with 240 impulses per second. Stimuli of higher frequency were ineffective. The inhibition of the bladder caused by stimuli within the most effective range usually outlasted the stimulation for a considerable time, sometimes for as much as 1-2 minutes or more. Lowering the frequency of the stimuli to 3.5/sec. caused a reversal of the response. A contraction occurred as seen at No. 9 and 10 in Fig. 2. The contractions induced did not persist during the whole period of stimulation, the pressure tending to decline to the initial level after the peak of the contraction was reached. The latent period for the excitatory as well as the inhibitory responses

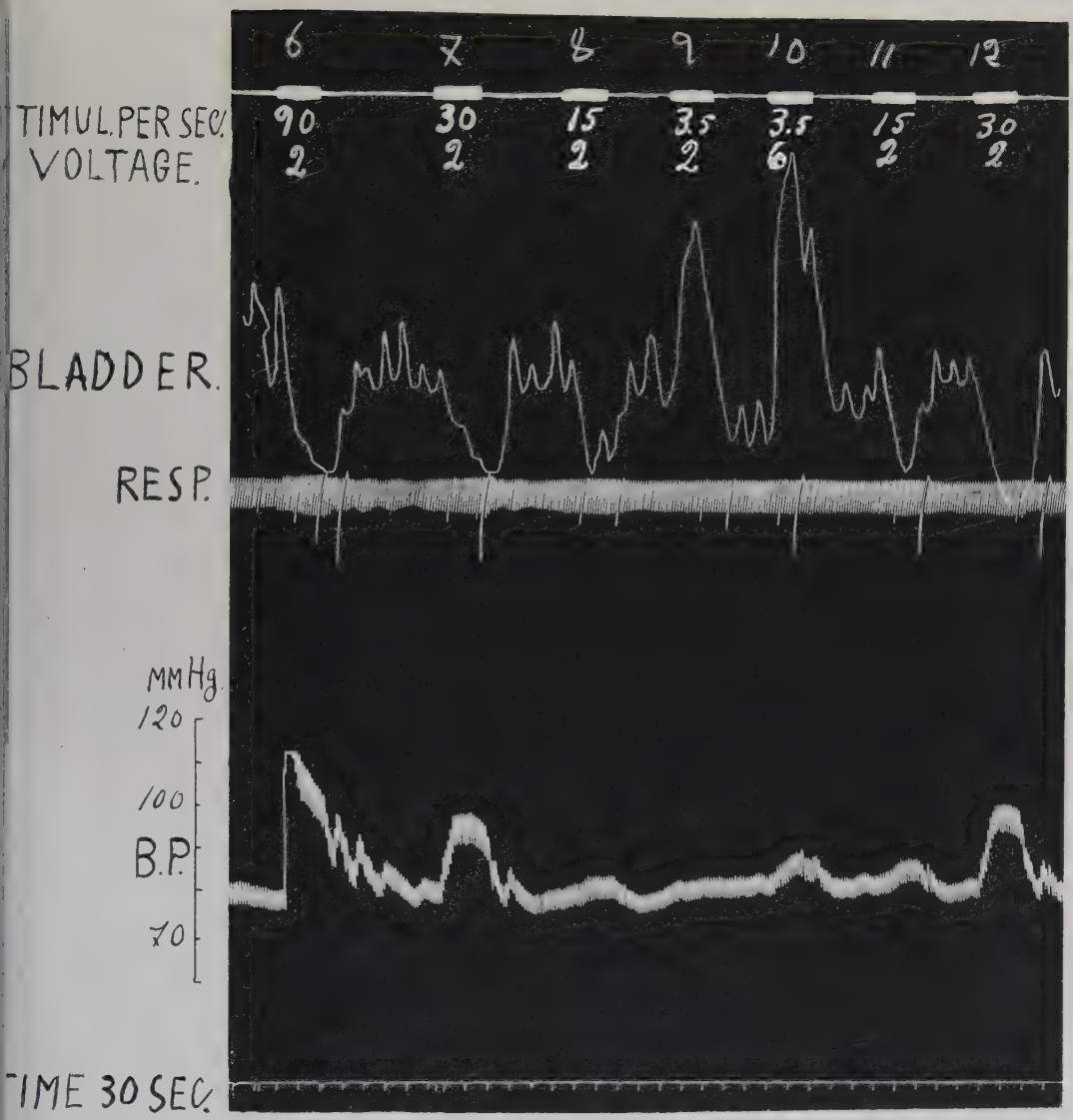

FIG. 2.

Alterations in bladder, blood pressure and respiration on stimulation of a point in the supraoptic region. Frequency varied.

was short, responses being regularly seen to start in a few seconds after the beginning of the stimulus. Fig. 3 illustrates the fact that points yielding bladder contractions will be missed unless stimuli of low frequency are used. As observed by other investigators no correlation was observed between the bladder, blood pressure and respiratory responses.

No definite conclusions can be drawn about the strict localization of the points concerned with bladder activity until a more complete microscopical investigation is made. From the material at hand points giving bladder contractions were found in the preoptic region as well as in different regions of the hypothalamus. Most of them however were concentrated in the anterior part of the hypothalamus and the posterior part of the preoptic region. Most of the excitatory responses could be reversed to inhibitory ones by increasing the frequency of the stimulus. In addition, numerous points were found

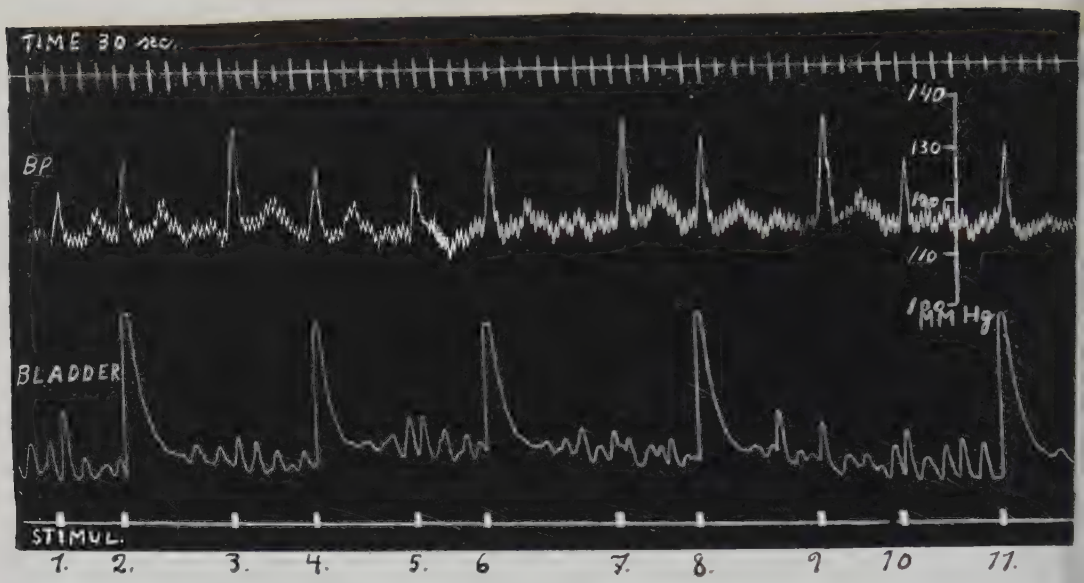

FIG. 3.

Alterations in bladder and blood pressure on stimulation of a point in the supraoptic region. Frequency and intensity of stimulus varied.

No.	Stimul./sec.	Voltage (V)	No.	Stimul./sec.	Voltage (V)
1.	7.5	0.1	3.	60	1.0
2.	"	1.0	5.	60	0.8
4.	"	0.5	7.	60	1.2
6.	"	0.8	9.	120	0.8
8.	"	1.2	10	60	0.8
11.	"	0.8			

scattered throughout the preoptic region, hypothalamus and upper mid-brain from which only inhibition could be elicited.

Discussion. The experiments have shown that the bladder response to hypothalamic stimulation is strongly influenced by the frequency of the stimulus. High frequency stimuli usually give inhibitory, low frequency stimuli usually give excitatory bladder responses. Most of the excitatory responses could be reversed to inhibitory ones by increasing the frequency of the stimulus. Whether the reversal effects are the result of the activation of different nervous structures or whether the same nervous elements are influenced in a different way by stimuli of different frequency or whether some other explanation is to be found, awaits further determination.

In the study of Kabat *et al.*,[3] Magoun[4] and Wang and Harrison[9] points giving inhibition of the bladder were only rarely seen, a fact probably due to the absence of bladder activity in their animals under barbiturate anesthesia. The inability to observe inhibition under these conditions together with the fixed frequency of the stimulus employed (Harvard inductorium), makes it apparent that these investigations did not give a complete picture of the localization of nervous structures concerned with bladder activity. It would seem necessary to reinvestigate this problem using stimuli of varying characteristics.

In their experiments on chloralosed cats Beattie and Kerr[5] reported that points yielding inhibition of the bladder were only found in the posterior part of the hypothalamus and the upper mid-brain. The present results, showing points giving inhibitory bladder responses scattered throughout the whole hypothalamic area, do not favor the view held by these authors that the hypothalamus can be divided in an anterior excitatory and a posterior inhibitory part.

Summary. The bladder response to hy-

[9] Wang, S. C., and Harrison, F., *Am. J. Physiol.*, 1939, **125**, 2.

pothalamic stimulation is strongly influenced by the frequency of the stimulus used. High frequency stimuli (60/sec. or more) usually cause bladder relaxation, low frequency stimuli (3.5/sec.) usually bladder contraction. A reversal of the bladder response is frequently obtained by changing the frequency of the stimulus.

Points yielding excitation and inhibition of the bladder are found scattered throughout the whole preoptic and hypothalamic area but points giving bladder contraction seem to be concentrated in the rostral parts of the diencephalon.

15741 P

Mitotic Activity in Hypophysis of Pregnant Rat after Injections of Estrogen.

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Division of cells by mitosis increases in the hypophysis of young mature rats in the late estrous phase of the sexual cycle.[1] That this is due to the increase in the level of estrogen is shown by the fact that mitoses can be increased by injections of estrogenic hormones.[2] Thus, 25 μg of estradiol benzoate (Progynon-B)* injected into 3-month-old ovariectomized rats 72 and 48 hours before death will result in an average mitotic activity of 22.09 mitoses per square mm of section. During pregnancy, however, even though there is a continued production of estrogenic hormone, mitoses are rare in the hypophysis after the 3rd day.[3] Just what level of estrogen occurs during pregnancy is not known, and it seems possible that either the level is not sufficiently high to cause an increased mitotic activity or that there is some substance that inhibits the mitosis-stimulating effect of the estrogen that is produced.

In order to test this point, a group of 22 pregnant animals approximately 3 months of age were injected with 25 μg of estradiol benzoate, 72 and 48 hours before death. In addition to these, 3 others that did not receive injections were killed on the 13th day of pregnancy to serve as controls. Of the pregnant animals that received injections of estradiol benzoate, 9 were killed on the 6th, 7th or 8th day of pregnancy and 13 on the 12th, 13th, 14th or 15th day. Of those killed early in pregnancy, 3 animals showed evidence that implantation had occurred but in 2 of these abortion had apparently occurred. Those killed later in pregnancy all had living fetuses.

To determine the mitotic activity, all mitotic figures were counted in a 3-micra coronal section of known area from each hypophysis.

The results are summarized in Table I. Animals receiving injections before implantation of ova (Group 1) have an average of 17.33 mitoses per sq mm. This is not significantly different ($P. > .4$) from the results obtained by injecting ovariectomized animals (Group 4),[2] and, as with the latter group, there is a considerable range in mitotic activity. The animal having the lowest count (3.3 mitoses per sq mm) was the only one that showed normal implantation sites.

Animals injected later in pregnancy and killed from the 12th to the 15th day have an average mitotic activity of 4.35 per sq mm (Group 2). Nine of the 13 animals have consistently low counts (average 1.5), the other 4 falling within the range found for

1 Hunt, T. E., *Anat. Rec.*, 1942, **82**, 263; *Endocrinology*, 1943, **32**, 334.

2 Hunt, T. E., *Anat. Rec.*, in press.

* The estradiol benzoate (Progynon-B) was kindly furnished by the Schering Corporation, Bloomfield, N.J.

3 Hunt, T. E., *Anat. Rec.*, 1943, **85**, 32.

TABLE I.
Mitotic Activity in the Hypophysis of Rats.

Group		No.	Age, avg and range, days	Mitoses per mm² Mean ± S.E.
1	Pregnant 6-8 days with estrogen	9	92 (89-93)	17.33 ± 3.27
2	Pregnant 12-15 days with estrogen	13	94 (83-106)	4.35 ± 1.41
3	Pregnant without estrogen	3	95 (94-98)	.72 ± .64
4	Ovariectomized with estrogen	10	83 (76-90)	22.09 ± 5.46

the injected ovariectomized Group 4. In comparing Groups 2 and 4, the difference is definitely significant statistically ($P. < .01$).

The 3 animals not receiving injections and killed on the 13th day have an average mitotic activity of .72. This is essentially the same as the low mitotic activity found previously in somewhat older animals after the 3rd day of pregnancy[3] and is also not significantly different from the average found in ovariectomized animals of the same age.[2]

The results show that during pregnancy something is produced that, in most cases, suppresses the mitosis-stimulating effect of estrogen. The fact that the suppression does not occur until after implantation suggests that the placenta produces the inhibiting substance. Since, in some cases, the mitotic activity in the hypophysis is not suppressed, it is necessary to assume either that the inhibiting effect may be overcome or that the cells of the hypophysis are less refractory to mitosis-stimulating substances in some animals.

15742

The Use of a Resistance Wire, Strain Gauge Manometer to Measure Intraarterial Pressure.

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For use in measurement of intraarterial pressure in man under ordinary circumstances in the laboratory or clinic, the high frequency, hypodermic manometer developed by Hamilton and his associates[1] has not been excelled in respect to its combination of accuracy and simplicity of operation. In certain unusual situations, however, this manometer is inconvenient to use, because it must be rigidly fixed with respect to the recording camera and records must be made within a few feet of the subject. Attempts to overcome these disadvantages have led to development of means of converting pressure to electrical energy,[2-4] so that arterial blood pressure might be recorded with more freedom at some distance from the subject by means of a galvanometer. The purpose of this paper is to describe the use of a resistance wire, strain gauge pressure transmitter to accomplish electrical translation in measurement of arterial blood pressure.

The strain gauge pressure transmitter*,[5] consists of a balanced Wheatstone bridge

[1] Hamilton, W. F., Brewer, G., and Brotman, I., *Am. J. Physiol.*, 1934, **107**, 427.

[2] Rein, H., *Arch. f. d. ges. Physiol.*, 1940, **243**, 329.

[3] Hampel, A., *Arch. f. d. ges. Physiol.*, 1940, **224**, 171.

[4] Lilly, J. C., *Rev. Scient. Instruments*, 1942, **13**, 34.

* Manufactured by Statham Laboratories, 8222 Beverly Blvd., Los Angeles, Calif.

[5] Meyer, R. D., *Instruments*, 1946, **19**, No. 3.

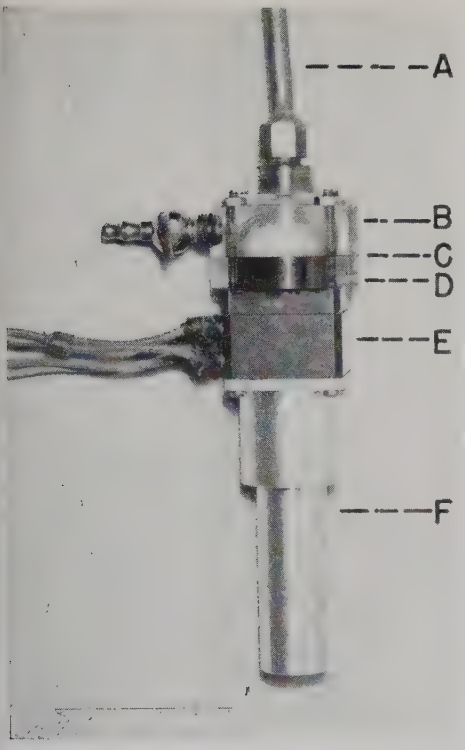

FIG. 1.
Strain gauge pressure transmitter adapted for measurement of arterial blood pressure. A. Lead tube to which needle is attached. B. Lucite chamber filled with anticoagulative solution. Stopcock for fluid reservoir. C. Plastic membrane (Koroseal). D. Lucite chamber filled with oil. E. Case containing strain gauge. Wires lead to battery and galvanometer. F. Holder.

whose 4 elements are made up of strain sensitive wire. The 4 wires are mounted on a cantilever suspension, movement of which increases the strain on one pair of wires and decreases it on the other. Since the resistance of the wires changes with variation in strain, movement of the suspension destroys the balance of the bridge and causes current to flow in the output circuit. The magnitude of the current is directly proportional to the amount of movement of the suspension. The bridge may be powered by dry cells (6 to 20 volts) and the output current measured by means of a microammeter or suitable recording galvanometer. No amplification is necessary. In utilizing this device as a pressure transmitter the cantilever suspension is connected to a bellows or diaphragm. Movement of the bellows or diaphragm caused by application of fluid or gas pressure alters the strain on the bridge circuit so that the amount of pressure applied can be determined by measuring the output current.

In order to adapt the pressure transmitter for use in measurement of blood pressure, the housing which surrounds the bellows through which pressure is transmitted to the strain sensitive wires has been replaced by a cylindric lucite chamber (Fig. 1). The latter is filled under vacuum with nonvolatile oil (evacuated hydraulic fluid) and is separated from a second lucite chamber by means of a loose plastic membrane (Koroseal). To the second chamber is attached a lead tube and needle used for arterial puncture and a stopcock which leads to a fluid reservoir. This system is filled with anticoagulative solution consisting of air-free solution of 0.9% sodium chloride containing 20 mg of sodium heparin per liter. Use of oil in the first chamber prevents corrosion of the metal bellows by the action of the anticoagulative solution. Isolation of the first chamber from the second by means of a plastic membrane eliminates the possibility that air bubbles may become lodged in the folds of the bellows whenever the manometer is filled and washed with anticoagulative fluid.

Strain gauge pressure transmitters which have several ranges of sensitivity are manufactured. The gauge which has been used for measurement of arterial blood pressure has a closed circuit output of about 14 microamperes for a pressure equivalent to 100 mm of mercury (bridge resistance 273 ohms) over a total range of $\pm$ 780 mm of mercury. With use of this gauge, a lead tube $\frac{1}{8}$ inch (0.3 cm) (inside diameter) by 18 inches

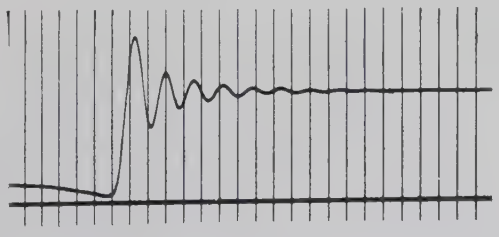

FIG. 2.
Natural frequency of strain gauge manometer with 18-inch lead tube and 19-gauge needle. Time interval is 0.01 second.

TABLE I.
Natural Frequency of Manometers Using Various Strain Gauge Pressure Transmitters.

Strain gauge No.	Full scale range, mm Mercury	Needle* gauge	Natural frequency, cycles per second	
			18-inch lead tube†	No lead tube
PV-107	± 780	19	63	78
		17	80	106
P6-8D-350	± 415	19	29	33
		17	33	50
P6-4D-250	± 208	19	20	25
		17	24	36

* 1¼ inches in length.
† ⅛ inch internal diameter.

(45.7 cm) and a 19-gauge needle 1¼ inches (3.2 cm) long, the natural frequency of the manometer is about 60 cycles per second (Fig. 2) as determined by the method described by Hamilton and his co-workers.[1] The displacement of fluid into the needle is about 0.5 cu mm when a pressure of 100 mm of mercury is applied.

More sensitive strain gauge pressure transmitters are available, such as those which have a total range of ± 415 mm of mercury or ± 208 mm of mercury, but their fluid displacement is greater and the natural frequency of the manometer using these gauges is considerably lower than that described in preceding sentences (Table I).

The galvanometer which has been used with the pressure transmitter is the Type A manufactured by the Heiland Research Corporation.† The natural undamped frequency of this galvanometer is 40 cycles per second. When critically damped, its deflection is within ± 3% of the true response up to a signal frequency of about 35 cycles per second but falls to 50% at about 60 cycles per second. With the galvanometer located 2 meters from the recording camera, the sensitivity of the entire manometer system is such that a deflection of 50 mm is equivalent to a pressure of 100 mm of mercury. The calibration of the system is strictly linear throughout its range and is stable so long as the voltage of the batteries is maintained. Hysteresis is approximately 0.5%. Moderate changes in temperature have only a negligible effect on the system.

† Heiland Research Corporation, 130 East Fifth Avenue, Denver, Colo.

With the exception that recording is done by galvanometer, the technics and precautions used in measuring pressure with the strain gauge manometer are the same as those employed with the Hamilton manometer.[1] However, because of the relatively large volume of fluid which enters the strain gauge manometer when pressure is applied, special care has been taken to avoid clotting of blood which enters the needle while measurement of blood pressure is being made. The bore of the stainless steel needle is polished by drawing through it a thread impregnated with thick grease containing fine emery dust. Before the needle is used, its inside surface, particularly near the point, is coated very thinly with paraffin. While the needle is in the artery, it is flushed periodically with a very small quantity of sterile anticoagulative solution. With these precautions, pressures have been measured continuously for more than an hour.

The disadvantage of the strain gauge manometer which has been described herein is the low natural frequencies of the manometer and the galvanometer. To determine the limitation which this may impose on use of the strain gauge manometer, the accuracy of this manometer for recording arterial blood pressure has been tested by comparison with a Hamilton manometer which had a natural frequency of 115 cycles per second. Simultaneous recordings obtained by use of the 2 manometers in studies on dogs have demonstrated that the strain gauge manometer accurately records normal peripheral pulses (Fig. 3), but that it may introduce instrumental errors in recording more abrupt cen-

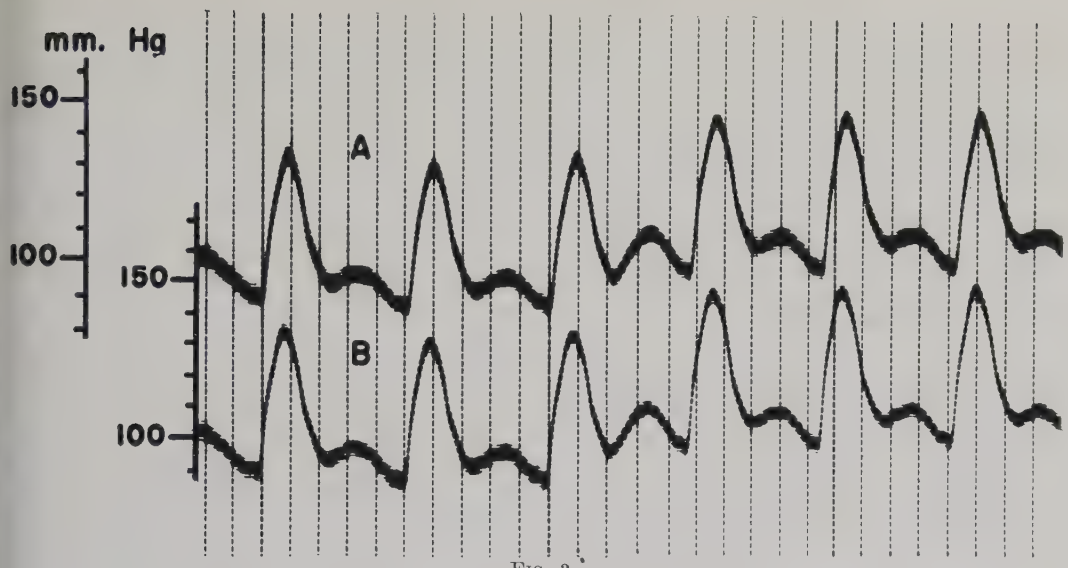

FIG. 3.

Femoral arterial blood pressure of a dog recorded simultaneously by Hamilton manometer (A) and strain gauge manometer (B). Time interval is 0.1 second.

tral pulses as, for example, those of the common carotid artery. For this reason the strain gauge manometer cannot be recommended for the study of arterial blood pressure and pulse wave forms in clinical investigations in which the Hamilton manometer can be employed.

To increase the natural frequency of the strain gauge manometer would require sacrifice of its sensitivity. Because of the limited sensitivity and the low frequency response of recording devices presently available, the use of electronic amplifiers would then be necessary to obtain satisfactory records. Other arrangements of strain sensitive, resistance wire designed for use with amplifiers have been employed in physiological research,[6] but their suitability for recording arterial blood pressure has not yet been reported.

In its present stage of development, however, the strain gauge manometer which has been described herein is useful for measuring the pressure in peripheral arteries in situations in which electrical translation is necessary. For example, this manometer has been

[6] Grundfest, Harry, Hay, J. J., and Feitelberg, Sergei, *Science*, 1945, **101**, 255.

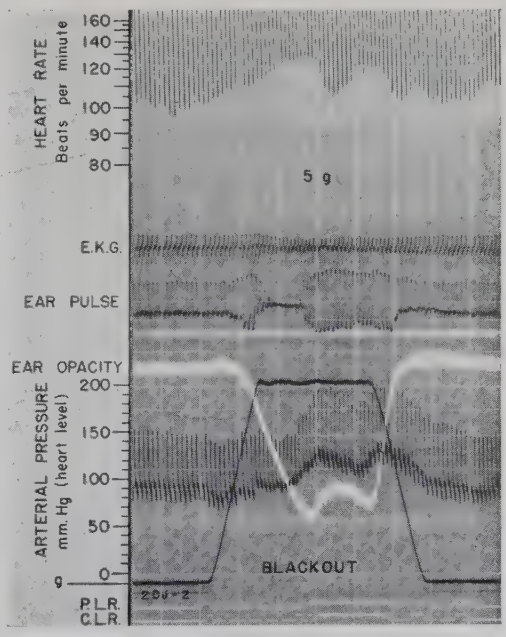

FIG. 4.

Arterial blood pressure and other physiologic variables in man during exposure to centrifugal force (g). Pressure measured in radial artery with wrist at heart level by means of strain gauge manometer. Vertical white lines are spaced at intervals of 5 seconds. P.L.R. and C.L.R. show subject's response to light signals in peripheral and central fields of vision.

useful in determining the changes in arterial blood pressure which occur in man during exposure to centrifugal force on a human centrifuge[7,8] (Fig. 4). In this case the output current of the strain gauge was carried from the rotating centrifuge through mercury ring contacts and recorded in another room about 60 feet from the subject.

Summary. The use of a resistance wire, strain gauge to convert pressure to electrical energy in making direct measurements of arterial blood pressure is described. The electrical circuits involved are simple. Recording is by means of a galvanometer without the use of electronic amplifiers. The calibration of the manometer is linear, stable and relatively insensitive to temperature changes. The natural frequencies of the manometer and galvanometer are 60 and 40 cycles per second, respectively.

Addendum. Manometers constructed with strain gauges of a more recent model (P6-15D-250, serial No. 275) have natural frequencies up to 100 cycles per second when fitted with an 18-inch (45.7 cm) lead tube and a 19-gauge needle. Their sensitivity has remained the same as that of previous strain gauge manometers.

7 Lambert, E. H., and Wood, E. H., *Fed. Proc.*, 1946, **5**, 59.

8 Wood, E. H., Lambert, E. H., Baldes, E. J., and Code, C. F., *Fed. Proc.*, 1946, **5**, 327.

15743

Sodium Cyanide: Time of Appearance of Signs as a Function of the Rate of Injection.*

ARTHUR A. WARD, JR. (Introduced by W. S. McCulloch.)

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The effects of sudden intravenous injections of NaCN in animals have been described elsewhere.[1] On the basis of both human and animal studies,[2] certain conclusions have been drawn as to the probable detoxification rate for this compound based on the assumption that this rate is constant. Because of possible therapeutic applications, it is important to determine in what manner the body responds to slow, constant infusions of dilute solutions of the sodium salt of cyanide, and whether the detoxification rate varies as a function of the rate of injection.

Method. Experiments were carried out on cats of about 2 kg paralyzed with dihydro-beta-erythroidine hydrobromide† (10 mg *i.v.* followed by about 0.1 mg per min. continuously) and maintained on artificial respiration at 25 strokes/min. of 35 cc/stroke. The skull was laid bare and the electrical activity of the cortex obtained with skull electrodes was recorded with a 6-channel, ink writing Grass oscillograph, the EKG being recorded with the same apparatus, using the conventional leads. Changes in the oxygen tension of the cortex were concurrently recorded.[3] NaCN was injected continuously‡ by an accurately calibrated 50 cc syringe driven by a synchronous motor, delivering from 0.033 ml/min. to 0.54 ml/min. By

* The work described in this paper was carried out under a contract between the Medical Division, Chemical Corps, U. S. Army, and the University of Illinois College of Medicine.

1 McCulloch, W. S., and Wheatley, M. D., in press.

2 Loevenhart, A. S., Lorenz, W. F., Martin, H. G., and Malone, J. Y., *Arch. Int. Med.*, 1918, **21**, 109; Loevenhart, A. S., Malone, J. Y., and Martin, H. G., *J. Pharm. and Exp. Therap.*, 1922, **19**, 13.

† The author wishes to thank Merck & Co. who kindly supplied this drug.

3 Davis, E. W., McCulloch, W. S., and Roseman, E., *Am. J. Psychiat.*, 1944, **100**, 825.

‡ The author wishes to thank Dr. R. K. Richards of the Abbott Laboratories who loaned to us this apparatus.

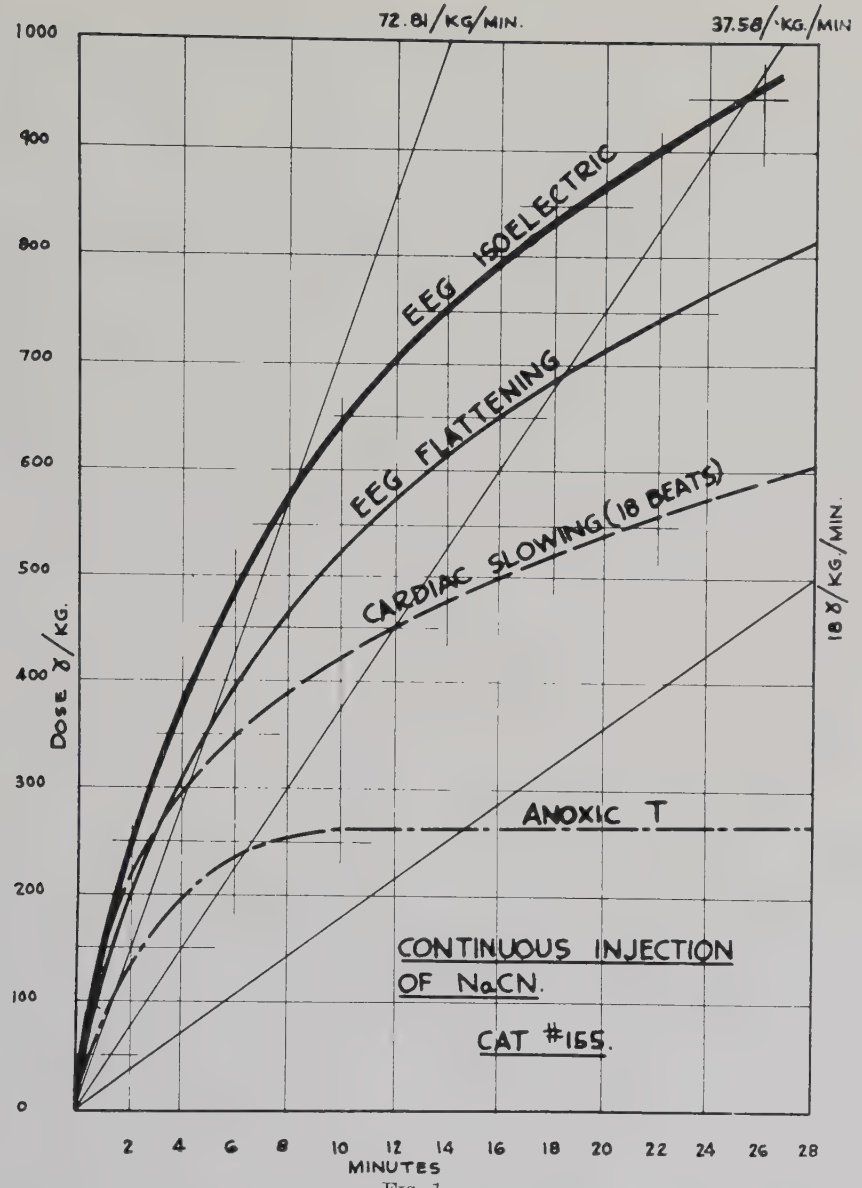

FIG. 1.

Continuous injection of NaCN at the rate of 18, 37, and 72 μg/kg/min. (diagonal straight lines). The time of appearance of the various phenomena is plotted against time at these various rates of injection (curved lines).

varying the concentration of NaCN in the solution, rates of injection from 7 μg/kg/min. to 100 μg/kg/min. could be achieved with accuracy over the entire range.

Results. The effects of slow, constant infusion of NaCN are similar to those which follow sudden injection,[1] the time of onset varying with the rate of injection. One of the first effects is a slight increase in both the frequency and amplitude of the electrical brain waves which is soon followed by the appearance of large, slow waves, with occasional superimposed bursts of fast, small waves. This is in turn followed by a gradual reduction in both the amplitude and frequency, ending, if the dose is great enough

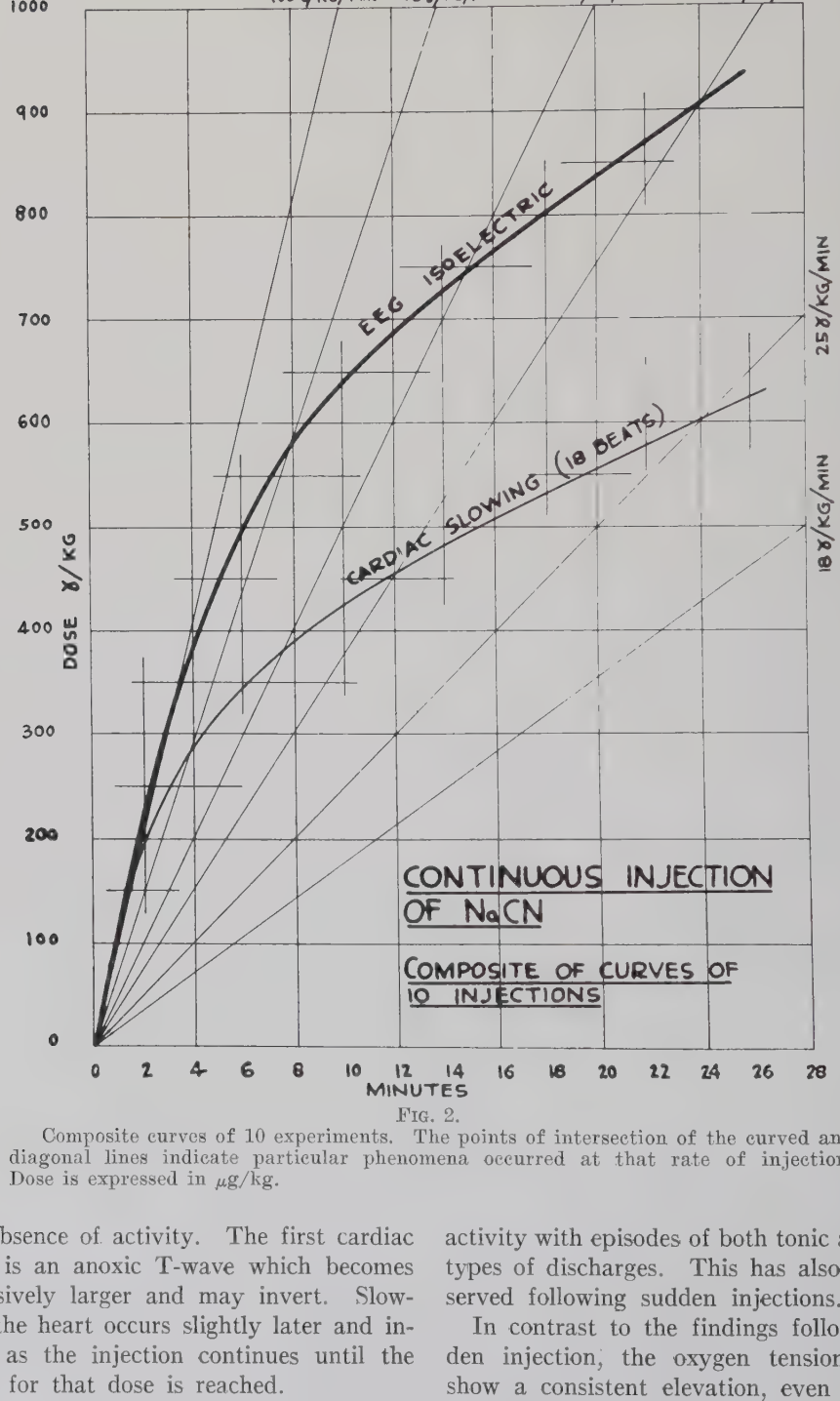

FIG. 2.

Composite curves of 10 experiments. The points of intersection of the curved and diagonal lines indicate particular phenomena occurred at that rate of injection. Dose is expressed in μg/kg.

in an absence of activity. The first cardiac change is an anoxic T-wave which becomes progressively larger and may invert. Slowing of the heart occurs slightly later and increases as the injection continues until the plateau for that dose is reached.

During the recovery from the effects of continuous injection, the electrical activity of the cortex often shows a period of hyperactivity with episodes of both tonic and clonic types of discharges. This has also been observed following sudden injections.

In contrast to the findings following sudden injection, the oxygen tension fails to show a consistent elevation, even when the EEG changes are most marked.

As an index of the action of NaCN, various clear and consistent phenomena have

been chosen as end-points, namely: onset of anoxic T-wave; cardiac slowing of 18 beats/min.; increase in frequency and also flattening of the electrical activity of the cortex; and onset of isoelectricity of the EEG. The onset of these, abstracted from the continuous records, are plotted on the straight lines representing the rates of injection. The points for each variety of end-point lie on curved lines. The complete curves for one representative experiment (Cat No. 155) are presented (Fig. 1) as well as one composite curve containing the average values for 10 animals (Fig. 2). It will be noted that while the various effects of NaCN occur serially close in time at high rates of injection, at slower rates of injection they are disproportionally spread out in time.

Discussion. It is not surprising that the oxygen tension is inconsistent when both the consumption and the supply are variably decreased.

If detoxification of NaCN in the body proceeded at a constant rate, the plots of the end-points of any variety would fall on a straight line. The observed ascending curves being convex upward, indicate that the rate of detoxification increases with the concentration—as it would if it followed the law of mass action. The clinical correlate of this concept is that a greater margin of safety obtains at slower rates of injection and for higher end-points. Thus in Fig. 1 there is an interval of 3 minutes between EEG isoelectricity and EEG flattening when NaCN is injected at 73 μg/kg/min. and an interval of 7 min. at 38 μg/kg/min. At slower rates the interval increases greatly and at 25 μg/kg/min. isoelectricity never occurs.

As the rate of injection is increased so as to approach the injection of the whole dose in 7 seconds, new factors begin to determine the time of appearance of the end-points. These include not only circulation time and time for diffusion into the tissues, but the time for the alteration of the tissue and the time for the development of the particular phenomenon as well as other things at present unknown. Any attempt to extrapolate from the curves for slow injection to the time of appearance of any sign after sudden injection is clearly unwarranted. It is important to note that these other factors determine delays too short to account for the observed curvature.

Summary. NaCN in dilute solution has been injected slowly at constant rates ranging from 7 to 100 μg/kg/min. The time and dose, at which several phenomena are first encountered, plot in ascending curves which are convex upward. This curvature probably indicates that detoxification of NaCN proceeds rapidly at higher concentrations.

15744

Effects of Use and Disuse on Nerve Endings, Neurosomes, and Fiber Types in Skeletal Muscle.*

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Pathologic neurosomes were first identified in the muscles of man[1] and monkey[2] during the early acute changes produced by poliomyelitis, and later in the muscles of rats and

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc., the Baruch Committee on Physical Medicine, and the Medical Department of Hoffmann-La Roche, Inc.

1 Carey, E. J., Massopust, L. C., Zeit, W., and Haushalter, E., *J. Neuropath. and Exp. Neurol.*, 1944, **3**, 121.

2 Carey, E. J., *Am. J. Path.*, 1944, **20**, 961.

chameleons during the onset of shock following hemorrhage,[3] heat,[4] trauma,[5] chemical action,[6] and histamine injection.[7] Suggestive evidence of the probable relation of the periodic secretion from the nerve endings to the granular and agranular muscle fibers, as well as to the clumping effect of DDT[8] on the neurosomes, had been demonstrated. The discharge of pathologic neurosomes into muscle during the early stages following nerve section[9] had likewise been shown, as well as the possible relation of the progressive loss of the normal, fine neurosomes to the gradual disappearance of the normal, dark and granular muscle fiber during atrophy. The purpose of this paper is the histologic demonstration of the giant fusiform neurosomes, discharged from nerve endings, retarded in rate of discharge, diffusion, and dissolution during the early stages of muscular atrophy of disuse following tenotomy of the innervated gastrocnemius muscle of the rat.

Methods. Under aseptic surgical technic, the Achilles tendon of the right gastrocnemius muscle was completely severed by transverse section from the calcaneus, and 3 mm of the distal end of the tendon were excised, in 150 white rats *(Mus norvegicus)*. The gastrocnemius muscle of the left leg was allowed to remain intact and used as a control of the effects of normal use. At 24-hour intervals the morphologic changes in the right gastrocnemius muscle were compared with the normally used muscle of the left side in each of 5 rats over a 30-day period. Segments of the control and experimental gastrocnemius muscle and sciatic nerve were simultaneously subjected to the same histologic technics. In 30 additional white rats, 2 were selected at 48-hour intervals following tenotomy. A ligature tied at the cut end of the muscle had at the free end of the ligature suspended weights that varied from 10 to 30 g depending upon the size of the muscle. The living muscle *in situ* was gradually restretched prior to excision and gold impregnation for 10- to 15-minute periods alternating with 5 minutes of rest for 3 to 5 hours.

The method of gold impregnation and teasing of whole muscle fibers, previously described,[1-9] was found superior to any other neurologic technic for the detection of the structural changes of the nerve endings in muscle. This method was checked against the current popular ones in which silver or methylene blue are used. The muscles were likewise stained with osmic acid, Sudan III, Sudan black, and Scharlach R, as well as with ordinary stains such as hematoxylin and eosine, after fixation with either formalin, Zenker's fluid, or other fixatives. Formalin produced chemical changes and altered critical features of the morphology of the neuromuscular apparatus. No alcoholic dehydrating agent was used in the gold method or teased whole mounts. The teasing of whole muscle fibers was a better technic than that of cutting the muscle into sections for observation of the whole neuromuscular apparatus and the anatomical relationships and changes of the epilemmal axon, hypolemmal axon, granules of Kühne, cross striations and the granular and agranular muscle fibers. The following experimental observations will be confined to the structure of nerve endings and muscle revealed by the gold technic.

Results. Effect of normal use on nerve and muscle. The normal muscle observed after gold impregnation and in teased whole muscle fibers had narrow, dark, and coarsely granular muscle fibers (Fig. 1) scattered among others of various diameters which were either finely granular or relatively

[3] Carey, E. J., Massopust, L. C., Zeit, W., Haushalter, E., and Schmitz, J., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 115.

[4] Carey, E. J., Massopust, L. C., Haushalter, E., and Zeit, W., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 121; *Am. J. Path.*, 1946, **22**, 175.

[5] Carey, E. J., Massopust, L. C., Zeit, W., Haushalter, E., and Schmitz, J., *J. Neuropath. and Exp. Neurol.*, 1945, **4**, 134; Carey, E. J., Massopust, L. C., Zeit, W., Haushalter, E., Hamel, J., and Jeub, R., *Am. J. Path.*, 1945, **21**, 935.

[6] Carey, E. J., *Am. J. Path.*, 1944, **20**, 341.

[7] Carey, E. J., unpublished observations.

[8] Carey, E. J., Downer, E. M., Toomey, F. B., and Haushalter, E., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 76.

[9] Carey, E. J., Massopust, L. C., Haushalter, E., Sweeney, J., Saribalis, C., and Raggio, J., *Am. J. Path.*, 1946, **22**, 1205.

FIG. 1 TO 3.

Photomicrographs × 150. Normal innervation, Fig. 1; discharge of giant, fusiform neurosomes from end plates 15 days after tenotomy, Fig. 2; and atrophy of nerve endings and muscle 30 days after tenotomy, Fig. 3, whole, teased, gastrocnemius muscle fibers, white rat. Legend: ne, nerve endings; GNS, giant fusiform neurosomes. Gold chloride technic.

agranular and light (Fig. 1 and 4). These dark fibers were designated as hyperchrysophilous and the light ones as hypochrysophilous and achrysophilous. There were multiple gradations of affinity for gold between the extremes. The nerve endings were usually retracted and deeply impregnated with gold (Fig. 1) in the hyperchrysophilous, dark muscle fibers. In the hypochrysophilous fibers the nerve endings were usually expanded (Fig. 1) and had a decreased affinity for gold. The granules of Kühne usually formed a dense rim around the retracted nerve endings whereas there was a quantitative diminution of these granules, to the point of complete depletion, around the extended branches of the expanded nerve endings produced by neuroprotoplasmic streaming. In a differential count of 5000 nerve endings the retracted endings varied in length from 20 to 40 μ and the expanded endings from 40 to 60 μ.

The normal muscle in cross section (Fig. 4) had relatively narrow fibers with coarse granules or neurosomes and the wide fibers contained medium-sized and fine granules. There were variations in the size of the granules, however, in the fibers of different diameters. In other locations in this same muscle, some of the wide fibers were relatively agranular. The axon, nerve endings, granules of Kühne, and granules in the muscle fiber, all had the same reaction to gold. The size and distribution of the granules were assumed to be related to the different degrees in the process of hydrolysis after the neurosomes were discharged into the muscle.

Effect of tenotomy on nerve and muscle. There was a progressive loss of the differential types of muscle fibers following tenotomy (Fig. 2 and 3). The characteristic normal dark type of granular muscle fiber was gradually lost until, on the 30th day (Fig. 3) following tenotomy, it was very infrequently found. There was, likewise, a great depletion (Fig. 3) of the nerve supply. In some places the hypolemmal axons of the nerve endings were completely absent (Fig. 3) and all that remained were clumps of sole plate nuclei. The structural expression of the dark, coarsely granular muscle fiber (Fig. 1 and 4) was determined by the presence of the attached and normally functioning muscle with its innervation. The nerve endings became uniformly and deeply impregnated with gold and fusiform in shape (Fig. 2) between the 10th and 20th days following tenotomy in the rat. These fusiform nerve endings were moulded by the progressive shrinkage of the muscle fiber due to loss of muscle substance.

On the 15th day (Fig. 2) of disuse atrophy following tenotomy, there could be seen manifestations of various stages in the discharge of giant fusiform neurosomes from the oblong and spindle-shaped nerve endings which were hyperchrysophilous. From the 3rd to the 15th day following tenotomy, in certain places in the muscle, a progressive increase in the number of the giant neurosomes was found. From the 15th to the 30th day following tenotomy, there was a progressive decrease in the number of these giant neurosomes. The discharged giant neurosomes were irregularly scattered in the myoplasm. Some of these giant fusiform neurosomes were uniformly and deeply impregnated with gold. Some were light in the center and dark at the tapering ends. Others were light at the ends and dark in the center. Still others were very faintly impregnated with gold, and their contained granules were arranged in cross striations undergoing progressive alignment with the cross striations of the muscle fiber.

These fusiform neurosomes varied from 10 to 275 μ in length and from 4 to 60 through the widest transverse diameter. In some places there were small oblong and fusiform neurosomes similar, in morphology and staining reaction to gold, to those observed during the early stages after nerve section in denervated muscle.[9] The large fusiform neurosomes produced a streamlining effect (Fig. 5 and 6) on the cross striations of muscle during the process of migration from the nerve ending and dispersion in the myoplasm of the muscle fiber. Some of these neurosomes in transverse sections were immediately under the sarcolemma whereas oth

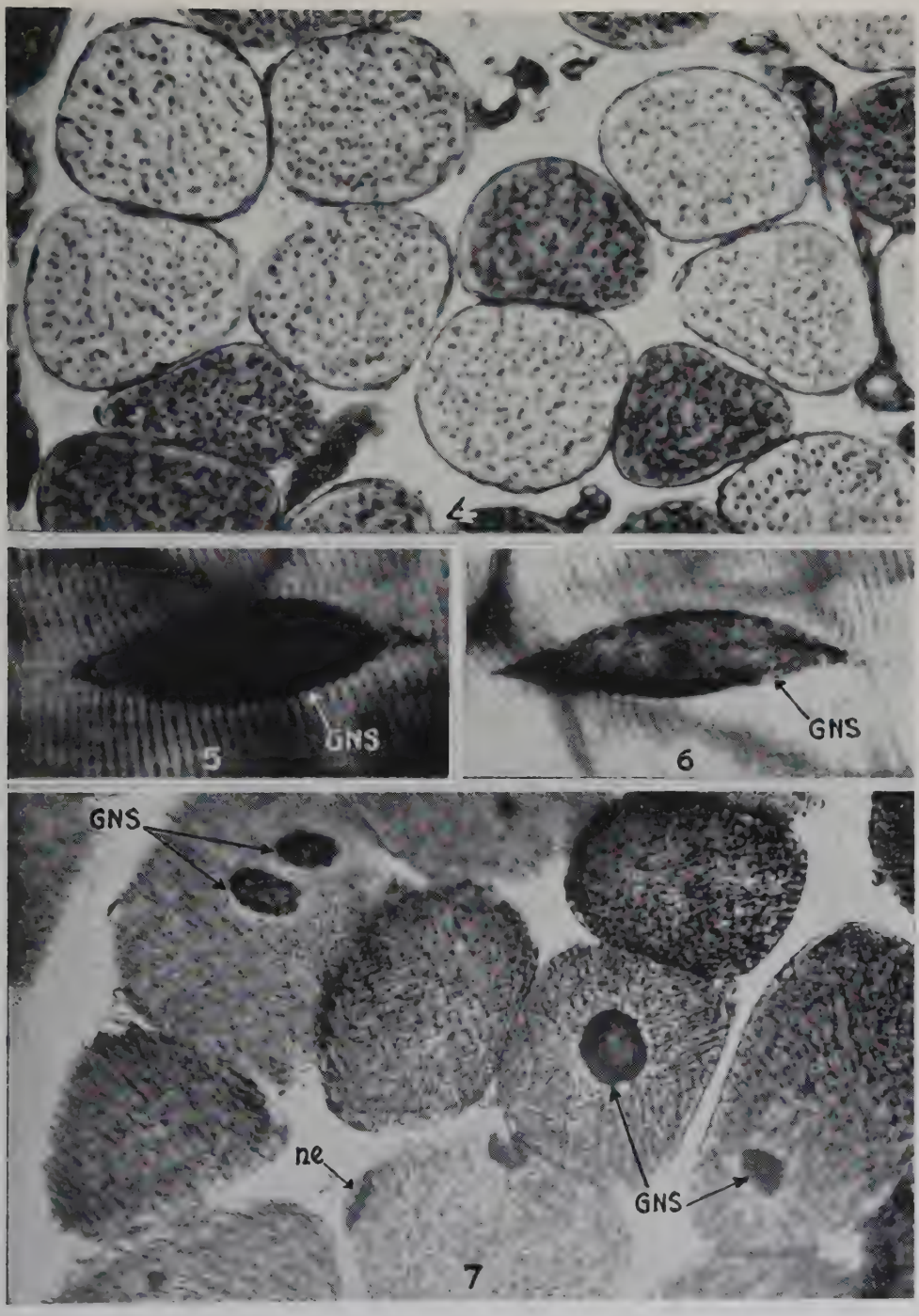

FIG. 4 TO 7.

Photomicrographs. Cross sections, normal, coarsely and finely granular muscle fibers × 400, Fig. 4; giant fusiform neurosomes in teased whole gastrocnemius muscle fibers × 600, Fig. 5 and 6; and cross sections of fiber containing giant fusiform neurosomes, gastrocnemius muscle, white rat, × 600, Fig. 7. Legend: ne, nerve endings; GNS, giant fusiform neurosomes. Gold chloride technic.

ers occupied the center (Fig. 7) of the muscle fibers. During disuse atrophy there was a progressive loss of the normal dark muscle fiber and of the normal coarse granules (Fig. 7) of the neurosomes. The granules were either very fine and dispersed, or aggregated into the giant fusiform neurosomes. In cross sections (Fig. 7) the giant neurosomes were either uniformly impregnated deeply with gold, or they had a light center or a rim of granules faintly reacting to gold.

The gastrocnemius muscle lost 30 to 50% of its weight, compared to the normal control, between 21 to 30 days after the tenotomy. There were individual variations in the rate of atrophy and in the morphologic changes of the neuromuscular apparatus and fibers of the gastrocnemius muscles. These variations were found not only in muscles from different animals examined after the same time interval following tenotomy, but also in different fibers in the same muscle. It was necessary, therefore, to survey great numbers of muscle fibers to detect the statistical trend of the morphologic changes.

The reestablishment of partial muscle stretch in some fibers of the muscle by regenerative attachment of the tendon to the subcutaneous tissue was a variable to be taken into consideration in evaluating the results. In some of the rats in our series the tendon of the muscle was freshly cut at 7-day intervals to eliminate the effects of partial reestablishment of stretch by union of tendon to subcutaneous tissue. The loss of normal muscle stretch determined the discharge of giant neurosomes. This was indicated by the total absence of the giant neurosomes in the series of 30 living muscles experimentally restretched *in situ* before excision and gold impregnation. Two muscles were restretched at 48-hour intervals until the 30th day after tenotomy.

Discussion. The basic mechanism in the physiology of use, increased use by exercise, and disuse, resulting, respectively, in muscle maintenance of health, hypertrophy, and atrophy of disuse and disease, is unknown. Young[10] stated that "the theories of the causes and nature of muscular atrophy are numerous but none is conclusive." This conclusion is held likewise by Carlson and Johnson.[11] The parts of the living body increase and decrease in size in proportion to the functional demand or use, but the essential underlying cause of these changes has remained an elusive one.

The hyperexcitability manifested by fibrillations may be prevented by quinidine, but Solandt and Magladery[12] observed that the denervated muscle continues to shrink in size. The slow, incoordinate activity of fibrillation, therefore, appears not to be the cause of the muscular atrophy. It has been demonstrated by Gutmann and Gutmann,[13] Hines,[14] Eccles,[15] and Solandt,[16] that the volume of denervated muscle may be fairly well maintained for a certain period by appropriate electrical exercises. Evidently, periodic optimum tension of traction and contraction (work) of muscles anchored to their attachments is necessary for muscle maintenance during health. Suggestive evidence was demonstrated previously that an optimum periodic tension of differential growth is necessary for the genesis of both smooth and skeletal muscle.[17] Evidence was also demonstrated that anatomic and experimental dampers to the lateral expansion of the stretched muscle fibers produces a replacement of muscle by fibrous tissue.[18]

The normal tension, or stretch, and work of the intact muscle attached to origin and

[10] Young, J. Z., *Lancet*, 1946, **2**, 109.

[11] Carlson, A. J., and Johnson, V., *The Machinery of the Body*, University of Chicago Press, Chicago, 1937, 620 pp.

[12] Solandt, D. Y., and Magladery, J. W., *Brain*, 1940, **63**, 255.

[13] Gutmann, E., and Gutmann, L., *Lancet*, 1942, **1**, 169.

[14] Hines, H. M., *J. A. M. A.*, 1942, **120**, 515; Hines, H. M., Thomson, J. D., and Lazere, B., *Arch. Phys. Therap.*, 1943, **24**, 69.

[15] Eccles, J. C., *J. Physiol.*, 1944, **103**, 253.

[16] Solandt, D. Y., de Lury, D. B., and Hunter, J., *Arch. Neurol. Psychiat.*, 1943, **49**, 802.

[17] Carey, E. J., *J. Gen. Physiol.*, 1920, **2**, 357; *J. Gen. Physiol.*, 1920, **3**, 61; *Am. J. Anat.*, 1921, **29**, 341; *J. Morphol.*, 1922, **37**, 1.

[18] Carey, E. J., *Am. J. Anat.*, 1936, **59**, 89.

insertion, appear to be necessary for the normal rate of discharge of granules from the motor end plates. This secretory process from the nerve endings may be slowed down by disuse of the innervated gastrocnemius muscle following tenotomy. By tenotomy, the normal stretch or tension of the muscle is destroyed. The lax muscle fibers released from one attachment are analogous to the broken strings of a violin. The detached strings are incapable of normal vibratory response because of loss of tone or tune. The rate of discharge of neurogenic substance into the muscle appears to depend upon the reciprocal interaction between nerve and muscle. The normal mechanical tension of the attached muscle fibers appears to determine the normal periodical flow of neurogenic substances into the muscle. The normally attached muscle fiber appears to act like an alternate pressure and suction chamber upon the nerve ending. This nerve ending appears to be a biological jet valve or ejector of the neurogenic secretion. Under normal conditions the rate of discharge, diffusion, and disappearance of neurosomes is excessively rapid. The release of normal muscle stretch by tenotomy decreases the demand, and slows down the rate resulting in accumulation of the neurogenic discharge into the abnormally flaccid muscle fibers.

Evidence now at hand supports the statement that the changes of fatty metamorphosis, Zenker's hyaline degeneration, the pathogenesis of dystrophy and of poliomyelitic muscle, are closely related to the pathology of the neuromuscular apparatus and alterations in the secretion of neurosomes into muscle. In the past, many of the fine physiologic neurosomes have been identified variously as the interstitial granules of Kölliker,[19] the J and Q granules of Holmgren[20] and the liposomes of Albrecht[21] and Bell.[22]

Evidence[1-7] had been accumulated and presented that the full fractional contraction, the relative relaxation, and the onset of contraction of functional activity, all were correlated with the types of nerve endings and muscle fibers. The dark, granular muscle fibers were proportionately increased in number by magnesium sulphate[9] and by agents that inhibited cholinesterase in its hydrolytic action on acetylcholine, such as thiamin chloride, atropine, curare, prostigmine, and ergotmine.[23] In high concentrations (0.25 to 0.5%) these chemicals injected into the living muscle increase proportionately by 25 to 50% the number of the dark granular muscle fibers.[23] On this incomplete evidence it is assumed that some of the granules in the dark muscle fibers are composed, at least in part, of acetylcholine.

The evidence presented in this paper supports the principle of the *Double Dependence* of nerve and muscle proposed by Young.[10] He stated that muscle receives its stimulation from nerve and exercises constraint against other muscles or outside forces. Muscle will atrophy if given too little direction from above, as after total denervation or isolation of lower from upper neurones; it will also atrophy if it is left relaxed by tenotomy and, therefore, cannot contract against resistance. We have observed, also, that disuse of the relaxed and attached gastrocnemius muscle, in the rat, by fixation of the whole limb in a cast produces changes in the neuromuscular apparatus similar to the effects of tenotomy.[24]

Summary. The limited experimental evidence presented in this paper tends to support the following statements:

The normally used and innervated gastrocnemius muscle of the white rat is characterized histologically by dark, coarsely granular and light, finely granular and

[19] Kölliker, A., *Z. f. Wissensch. Zool.*, Leipzig, 1857, **8**, 311.

[20] Holmgren, E., *Anat. Anz.*, 1907, **31**, 609; *Arch. f. mikr. Anat.*, 1907-08, **71**, 165; *Ibid.*, 1910, **75**, 240; *Anat. Anz.*, 1913, **44**, 225; *Nevraxe*, 1913, **14-15**, 277.

[21] Albrecht, E., *Verhandl. d. deutsch, path. Gesellsch.*, 1903, **6**, 63.

[22] Bell, E. T., *Anat. Rec.*, 1910, **4**, 199; *Internat. Monatschr. f. Anat. u. Physiol.*, 1911, **28**, 297; *J. Path. and Bact.*, 1912-13, **17**, 147.

[23] Carey, E. J., unpublished observations.

[24] Carey, E. J., unpublished observations.

agranular muscle fibers. The nerve endings are usually retracted in the dark, coarsely granular fiber and expanded in the light, agranular fiber, as revealed by gold impregnation of teased whole muscle fibers. The atrophy of disuse following tenotomy of the gastrocnemius muscle with nerve supply intact, is accompanied, during the first month, by the progressive loss of the narrow and dark, coarsely granular muscle fiber, and by a depletion of its innervation. The dark, granular muscle fiber is determined by the reciprocal interaction of the normally intact and attached muscle fiber with its normally functioning innervation. During the process of atrophy of disuse after tenotomy, small and giant fusiform neurosomes are discharged from the altered nerve endings. It is assumed that these giant fusiform neurosomes are the product of a retardation in the rate of the discharge, diffusion, and disappearance by hydrolysis, following tenotomy and disuse atrophy of the innervated gastrocnemius muscle. There appears to be a parallelism between the atrophy by disuse of tenotomized muscle and the loss of the normal discharge of neurosomes from the altered and progressively depleted innervation of the muscle. One factor in the atrophy of disuse of muscle appeared, therefore, to be the substantial loss of the discharge of neurosomes into muscle as well as the quantitative decrease of the myoplasm. The giant fusiform neurosomes that appear during the early period following tenotomy disappear when the living muscle *in situ* is adequately restretched prior to excision and gold impregnation.

15745

Ornithosis in Sea-Shore Birds.*

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Psittacosis-like infections have been reported in a large variety of wild and domesticated birds including parrots, finches, pigeons, chickens and turkeys.[1] Because of the wide prevalence of psittacosis-like infection among many species of wild and domesticated birds, Meyer proposed that the more general term "ornithosis" be employed in referring to nonpsittacine infections. It has not yet been found in partridges, quail or wild ducks.[2] Among the sea birds, the Fulmar petrel has been incriminated as the source of an epidemic of psittacosis in human inhabitants of the Faroe Islands.[3] Many of the avian species in which this disease is present ordinarily do not manifest clinical symptoms of the disease but carry the virus in "silent" fashion. This dormant infection of birds appears to be provoked to activity by conditions associated with crowding, malnourishment, or other effects of commercial aviary mismanagement. The disease might also be regarded as a natural population controlling factor, remaining inapparent until overpopulation and undernourishment permit the infection to become activated and aggravated in virulence.

Psittacosis (ornithosis) is an important public health problem; so it is important that the natural or potential reservoirs of the disease be known. A study of the incidence of ornithotic infections among sea shore birds

* Sincere appreciation is expressed to Drs. R. W. Strandtmann and C. U. Dernehl for their assistance in the capture and identification of the birds used in this study.

1 Meyer, K. F., Psittacosis and Ornithosis, in *Diseases of Poultry*, The Collegiate Press, Inc., Ames, Iowa, 1944.

2 Eddie, B., and Francis, T., Jr., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 291.

3 Rasmussen, R. F., *Zentralbl. f. Bakt.*, I Abt., Orig., 1938, **143**, 89.

has been initiated in our laboratories, with results which indicate that the infection prevails among them.

There are two methods which may be employed for the detection of ornithotic infections in birds: one involves the inoculation of mice with the suspected avian spleen, liver and kidney tissue emulsions; the other method involves the serological detection of infection with the complement-fixation technic. That the latter technic is accurate is affirmed by the extensive data presented by Bedson,[4] and by Meyer *et al.*[5] Francis and Gordon[6] increased the specificity of the antigen by preparing it from the chorio-allantoic fluid of the infected chick embryo.

Methods. Antigen. A standard strain of ornithosis virus (P-4), obtained from Dr. J. E. Smadel, was propagated in the chorio-allantoic fluid of 7-day-old chick embryos. After 5 days further incubation at 35°C, the extra embryonic fluids were collected from the eggs and this material was then inactivated at 56°C/30 minutes. The agent was sedimented in an angle head centrifuge at 6000 r.p.m./1 hour after which the sediment was resuspended in saline to 1/10 its original volume. It was then titrated with a known potent antiserum and its complement fixing titer was set at 2 units. This antigen was prepared by us while at the 8th Service Command Laboratory, Fort Sam Houston, Texas, and was subsequently preserved in the lyophilized state at refrigerator temperature until used in this problem.

Serum. The blood specimens were collected by heart puncture and after storage overnight in the refrigerator, the serum was separated and inactivated at 56°C/30 minutes. Specimens of serum were collected from the following species of birds: Laughing gull *(Larus atricilla L.)*, Royal tern *(Sterna maxima B.)*, Least tern *(Sterna antillarum L.)*, Common tern *(Sterna hirundo L.)*, Skimmer *(Rynchops nigra L.)*, Willet *(Catotrophorus semipalmatus G.)*, Gull billed tern *(Gelochelidon nilotica L.)*, Glossy ibis *(Plegadis autumnalis L.)*, Reddish egret *(Dichromanassa rufescens)*, Brown pelican *(Pelecanus occidentalis L.)*, Sooty tern *(Sterna fuscatus)*, and Sanderling *(Calidris leucophaea P.)*.

Test. The inactivated serum was diluted in saline serially from 1:5 to 1:160 in 0.2 cc amounts, 2 units of antigen, and 2 units of complement was added, and this mixture was incubated at 37°C/1 hour. The amboceptor and 2% sheep red blood cells were then added and further incubated at 37°C/30 minutes. At the end of this period the results were recorded, providing the serum, antigen and complement controls were satisfactory.

Results. It may be noted in Table I that 40% or more of the laughing gull, skimmers and willets showed complement-fixing antibodies for ornithosis in their blood serum. There was a lower incidence of antibodies in the blood serums of the terns and sanderlings, although the total numbers of the species were smaller. The majority of serum titers were in 1:5 and 1:10 dilutions; however, some went as high as 1:80 and 1:320 dilution. Of 165 birds of all species examined, 61 (36%) showed evidence of complement-fixing antibodies for ornithosis in the blood serum.

This serological evidence must necessarily be confirmed by actually demonstrating the agent in organs of naturally infected birds. Thus far, a virulent ornithosis-like agent has been isolated in mice from the pooled livers and spleens of 2 willets. This agent (W-ornithosis strain) is lethal for mice by intracerebral and intraperitoneal routes; in either case, nests of elementary bodies can be observed in the mononuclear cellular exudate of the spleen and meninges. The agent kills 7-day-old chick embryos in 4 to 5 days, in which case the chorio-allantoic fluid contains numerous minute elementary bodies. An antigen was prepared from the chorio-allantoic fluid, as described for the P-4 psittacosis antigen above, and it fixed complement with psittacosis serum, lymphogranuloma venereum serum, and its homologous rabbit serum. This latter serum also fixed

4 Bedson, S. P., *Brit. J. Exp. Path.*, 1936, **17**, 109.

5 Meyer, K. F., and Eddie, B., *J. Inf. Dis.*, 1939, **65**, 225.

6 Francis, R. D., and Gordon, F. B., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 270.

TABLE I.
Ornithosis in Seashore Birds by Complement Fixation Test.

Species	No. Tested	No. AC	No. +	% +	Complement fixation serum titers 1:5	1:10	1:20	1:40	1:80	1:320
Laughing gull	60	3	24	40	10	8	1	2	2	1
Willet	37	3	17	45.4	7	6	4			
Skimmer	15	0	8	53	4	2	2			
Sanderling	8	1	4		4					
Royal tern	15	0	1	6			1			
Least tern	8	0	2			1		1		
Common tern	6	0	3		1	11				
Gull billed tern	6	0	1	16		1				
Ring billed gull	1	0	0							
Glossy ibis	1	0	1						1	
Brown pelican	4	1	0							
Sooty tern	1	0	0							
Reddish egret	3	3	0							
	165	11	61	36	26	20	8	3	3	1

complement with lymphogranuloma venereum antigen and with psittacosis antigen.

The significance of finding evidence of ornithosis in sea-shore birds serves to add to the spectrum of information regarding the distribution of this disease in nature. While it may not be a great hazard to the human population, since these sea-shore birds are not generally confined in close association with man, nevertheless, this disease appears to exist in a multitude of avian species. Under proper circumstances these infected birds might contribute directly, or through other birds, to the morbidity rate of human ornithosis.

Summary. Serological evidence of ornithosis-like infections among several species of sea-shore birds is presented. Over 40% of the serums of sea gulls, willets, and skimmers which were examined showed complement-fixing antibodies for ornithosis. One strain of an ornithosis-like agent has been isolated from the willet species.

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Specific Complement-Fixing Diagnostic Antigens for Colorado Tick Fever.

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In the past Colorado tick fever[1,2] was believed to be a mild form of Rocky Mountain spotted fever.[3] However, largely due to the work of Florio and his colleagues,[4,5] it is now known that Colorado tick fever is not a rickettsial infection but a viral disease,[5] presumably tick-borne,[6] which apparently is quite distinct from Rocky Mountain spotted fever[1,3,7] and dengue fever[8,9] although resembling the latter both clinically and hematologically.

1 Becker, F. E., *Colorado Med.*, 1930, **27**, 36, 87.

2 Toomey, N., *Ann. Int. Med.*, 1931-32, **5**, 585.

3 Florio, L., Mugrage, E. R., and Stewart, M. O., *Ann. Int. Med.*, 1946, **25**, 466.

4 Florio, L., Stewart, M. O., and Mugrage, E. R., *J. Exp. Med.*, 1944, **80**, 165.

5 Florio, L., Stewart, M. O., and Mugrage, E. R., *J. Exp. Med.*, 1946, **83**, 1.

6 Topping, N. H., Cullyford, J. S., and Davis, G. E., *Publ. Health Rep.*, 1940, **55**, 2224.

7 Shaffer, F. C., *Colorado Med.*, 1935, **32**, 226.

8 Florio, L. Hammon, W. McD., Laurent, A., and Stewart, M. O., *J. Exp. Med.*, 1946, **83**, 295.

In previous communications submitted from this laboratory[10-12] a method was described for preparing specific diagnostic antigens for certain rickettsial and neurotropic viral diseases. The adaptation by Koprowski and Cox[13,14] of Colorado tick fever virus to the brain tissue of the laboratory mouse provided means of obtaining large amounts of infectious material for the preparation of complement-fixing antigen. The present work deals with the application of the above cited method in the preparation of complement-fixing antigen for the diagnosis of Colorado tick fever.

Materials and Methods. Virus Strains. Three strains of Colorado tick fever virus—the Florio (Fl), the Baker (B), and the Condon (C)—originally obtained from Dr. Lloyd Florio,*,[4] and adapted to the mouse brain in this laboratory,[13,14] were used for the preparation of the complement-fixing antigen. The Fl strain had undergone 30 hamster[4] and 35 mouse brain passages.[13] The B strain had been carried through 5 hamster[4] and 10 mouse brain passages,[14] while the C strain was adapted directly from the serum of a naturally infected human case to the brain of the dilute brown agouti (dba) mouse,[14] following which it was carried through 10 brain-to-brain passages in Swiss albino mice.

Stable virus preparations were readily obtained by preparing 5 or 10% infected brain suspensions in a 50-50 mixture of normal rabbit serum-saline (pH 7.4) and storing in the dry ice chest.

Antigens. Three groups of 300, 21-day-old Swiss albino mice were injected intracerebrally with 0.03 ml of a 1:50 dilution of infectious mouse brain suspension of the Fl, B and C strains of virus, respectively. Ten percent normal rabbit serum in saline was used as diluent. Four days later the animals in all 3 groups showed nervous symptoms but relatively little paralysis. The brains were removed aseptically from all mice of each group and antigens were prepared by lyophilization followed by benzene extraction, as previously reported by De-Boer and Cox.[11,12] Prior to processing, each lot of infected mouse brain suspension was tested for infectivity by intracerebral inoculation in 21-28-day-old Swiss albino mice. LD_{50} titers ranging from 10^{-6} to approximately 10^{-7} were obtained.

Immune serum. The Fl strain was used for the production of all immune serum by hyperimmunizing mice. Swiss albino mice, approximately 2 months old, were injected intraperitoneally with 0.5 ml of a 1:50 dilution of infected mouse brain suspension in distilled water. Thereafter, the mice were reinjected at weekly intervals with 0.5 ml of a 10% brain suspension until tests conducted with serum obtained from trial bleedings indicated that a satisfactory antibody titer was secured. A total of 10 injections was given before the mice were exsanguinated and their sera used for the tests reported herein. In addition several serum samples from clinically diagnosed human cases of Colorado tick fever which occurred in Colorado, obtained through the courtesy of Drs. Lloyd Florio and H. L. Morency,† were subjected to the tests.

Complement-fixation Tests. Complement-fixation tests were carried out identically to those reported previously.[12] Antigens were titrated for antigenic activity in the presence of various dilutions of homologous immune sera. The dilution of antigen giving the highest titer for the immune serum was used

9 Pollard, M., Livesay, H. R., Wilson, D. J., and Woodland, J. C., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 396.

10 Wolfe, D. M., Van der Scheer, J., Clancy, C. F., and Cox, H. R., *J. Bact.*, 1946, **51**, 247.

11 De Boer, C. J., and Cox, H. R., *J. Bact.*, 1946, **51**, 613.

12 De Boer, C. J., and Cox, H. R., *J. Immunol.*, 1947, **55**, 193.

13 Koprowski, H., and Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 320.

14 Koprowski, H., and Cox, H. R., in manuscript. Presented before the American Society of Tropical Medicine, Miami, Florida, November 5, 1946.

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† City Health Officer, Boulder, Colo.

TABLE I. Titration of Colorado Tick Fever Antigen.

Antigen	Dilution	Immune mouse serum, Serum dilutions 1:4	1:8	1:16	1:32	1:64	1:128	1:256	Controls, Serum dilutions 1:4	1:8	1:16	Antigen	Red blood cells	Hemolytic system
Fl	1:1	4	4	4	4	2	0	0	0	0	0	0	4	0
	1:2	4	4	4	4	1	0	0				0		
	1:3	4	4	4	4	±	0	0				0		
	1:4	4	4	4	2	0	0	0				0		
	1:8	4	4	±	±	0	0	0				0		
B	1:1	4	4	4	4	0	0	0	0	0	0	0	4	0
	1:2	4	4	4	4	1	0	0				0		
	1:3	4	4	4	4	1	0	0				0		
	1:4	4	4	4	4	2	0	0				0		
	1:8	4	4	2	0	0	0	0				0		
C	1:1	4	4	4	4	0	0	0	0	0	0	0	4	0
	1:2	4	4	4	4	±	0	0				0		
	1:3	4	4	4	4	1	0	0				0		
	1:4	4	4	4	4	1	0	0				0		
	1:8	4	4	4	2	0	0	0				0		

4 = complete fixation of complement. 3, 2, 1 = proportionately lesser fixation. ± = trace. 0 = no fixation.

in subsequent complement-fixation tests. Although this may require more frequent rechecking of the titer of an antigen, it is of value in obtaining the optimal titers of unknown sera. Complement was always titrated in the presence of antigen thus diluted, increasing the amount of 1:30 dilution of complement by 0.025 ml instead of 0.05 ml, thereby obtaining a more precise and sensitive test.

Neutralization Test. Equal volumes of undiluted test serum and aliquots of serial 10-fold dilutions of mouse brain suspension infected with either Fl or C strains of Colorado tick fever virus were mixed, incubated in a water bath at 37°C for 2 hours, and inoculated into 21-28-day-old Swiss albino mice by the intracerebral route. Normal human serum was used as control.

Experimental. Titration of Colorado Tick Fever Antigens. Table I shows the results obtained when the antigens prepared from Fl, B and C strains were titrated in the presence of an immune mouse serum prepared with Fl strain. It is apparent that all 3 antigens gave approximately the same degree of fixation of complement with the Fl serum.

In numerous additional tests it was determined that the benzene extracted antigens prepared from each of the 3 strains of Colorado tick fever virus consistently gave negative results in the presence of highly positive Wassermann human sera. These results thus confirm and extend the observations previously reported by DeBoer and Cox[11,12] in that benzene-extracted antigens are highly specific and do not give false positive reactions in the presence of markedly positive syphilitic sera.

Cross-fixation Tests with Colorado Tick Fever Immune Serum and Heterologous Antigens. To check the antigenic identity of the virus, Colorado tick fever immune mouse serum was tested against a number of viral and rickettsial antigens by the complement-fixation reaction. The Eastern (EEE) and Western (WEE) equine encephalomyelitis antigens were derived from infected chick embryos; the murine (endemic) typhus,

TABLE II.

Complement-fixation Tests with Colorado Tick Fever Immune Serum and Various Viral and Rickettsial Antigens.

	CTF* immune mouse serum							Controls					
	Serum dilutions							Serum dilutions					
Antigen	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:2	1:4	1:8	Antigen	Red blood cells	Hemolytic system
Western E.E.	0	0	0	0	0	0	0	0	0	0	0	4	0
Eastern E.E.	0	0	0	0	0	0	0				0	4	0
St. Louis	0	0	0	0	0	0	0				0	4	0
Japanese B	±	0	0	0	0	0	0				0	4	0
Rabies	0	0	0	0	0	0	0				0	4	0
Murine typhus	0	0	0	0	0	0	0				0	4	0
RMSF†	0	0	0	0	0	0	0				0	4	0
CTF*—F1	4	4	4	4	4	2–	0				0	4	0
CTF*—B	4	4	4	4	4	2	0				0	4	0
CTF*—C	4	4	4	4	4	1	0				0	4	0
American Q	0	0	0	0	0	0	0				0	4	0

* CTF = Colorado tick fever. †RMSF = Rocky Mountain spotted fever.

TABLE III.

Complement-fixation Tests with Colorado Tick Fever Human Sera.

			Human sera							Controls						
			Serum dilutions							Serum dilutions						
Serum	Blood taken* months	Antigen	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:1	1:2	1:4	1:8	Antigen	Red blood cells	Hemolytic system
No. 1	33	CTF—F1	±	0	0	0	0	0	0	0	0	0	0	0	4	4
No. 2	2½	CTF—F1	4	4	4	4	±	0	0	4	2	0	0	0	4	0
No. 3	2½	CTF—F1	4	4	4	±	0	0	0	4	4–	0	0	0	4	0
No. 4	42	CTF—F1	±	0	0	0	0	0	0	0	0	0	0	0	4	0
No. 5	7	CTF—F1	4	4	4	1	0	0	0	0	0	0	0	0	4	0
		RMSF	±	0	0	0	0	0	0					0	4	0
		Kolmer	0	0	0	0	0	0	0					0	4	0
No. 6	34	CTF—F1	4	4	4	1	0	0	0	0	0	0	0	0	4	0
		CTF—B	4	4	4	0	0	0	0					0	4	0
		CTF—C	4	4	4	0	0	0	0					0	4	0
		RMSF	0	0	0	0	0	0	0					0	4	0
		Kolmer	0	0	0	0	0	0	0					0	4	0

* Counting from day when diagnosis of Colorado tick fever was made.

Rocky Mountain spotted fever (RMSF) and American Q fever antigens were prepared from infected yolk sacs; while the St. Louis and Japanese B encephalitis and rabies antigens originated from infected mouse brains.‡ The antigens fixed complement in the presence of their homologous antisera in the following dilutions: EEE 1:64; WEE 1:64; murine typhus 1:128; RMSF 1:16; American Q 1:1024; St. Louis 1:64; Japanese B 1:64, and rabies 1:128.

The results obtained in testing the above antigens in the presence of a Colorado tick fever immune mouse serum are shown in Table II. No fixation of complement was obtained with any of the heterologous antigens, although all 3 Colorado tick fever antigens showed good fixation titers.

Complement-fixation Tests with Convalescent Sera. Table III summarizes the results of complement-fixation tests with Colorado tick fever antigen and human sera obtained at different intervals after the onset of the disease.

Serum No. 1 was taken from a person who had suffered a natural infection 33 months previously. Sera No. 2 and No. 3 were taken 2½ months after experimentally induced infections and were stored in the frozen state for 42 months. Serum No. 4 was from the same person as No. 3 but taken 42 months after the original infection and 30 months after another injection of virulent material. Sera No. 5 and No. 6 were from persons who had had natural infections 7 and 34 months prior to bleeding, respectively. These sera were tested for complement-fixing antibodies in the presence of Colorado tick fever, Rocky Mountain spotted fever and Kolmer Wassermann antigens.

The results shown in Table III indicate that sera No. 1 and No. 4 taken 33 and 42 months respectively after an attack of the disease, demonstrated only traces of complement-fixing antibodies in the presence of Colorado tick fever antigen. Sera No. 2 and No. 3 taken 2½ months after diagnosis of illness, and No. 5 and No. 6 taken 7 and 34 months, respectively, apparently showed significant antibody titers. The specificity of the test is again demonstrated by the fact that sera No. 5 and No. 6 showed fixation only with Colorado tick fever antigen and not with Rocky Mountain spotted fever, nor Kolmer Wassermann antigens.‡ In addition, it is seen that serum No. 5 fixed complement equally well in the presence of Colorado tick fever antigens Fl, B and C. This latter observation would seem to indicate that these 3 strains of Colorado tick fever are very similar antigenically, if not identical.

Comparison of Complement-fixing and Neutralizing Antibodies in Human Convalescent Sera. Table IV shows the results obtained in testing Colorado tick fever convalescent sera for their complement-fixing and neutralizing antibody titers. Since the serum samples were taken at various intervals from infected human cases, this test furnishes additional information regarding the time of appearance of complement-fixing or neutralizing antibodies and the length of time they persist during the convalescent period.

The results of the neutralization tests were in close agreement with those of the complement-fixation tests in all cases where the sera were not anticomplementary. Sera No. 2 and No. 3 were anticomplementary in dilution 1:1 and 1:2 to 2+ and 4+, respectively, which may account for the relatively higher complement-fixing titers (see Tables III and IV).

There is also close correlation in the results obtained with the 2 types of tests performed on sera of patients Ca, Og, Mo and Wi. For instance, the Ca serum, obtained 2 days after the diagnosis of Colorado tick fever, was devoid of all complement-fixing or neutralizing power. On the other hand, the Og serum taken on the 9th day after the disease was diagnosed, showed some complement-fixing and neutralizing capacity. Mo and Wi sera had definite complement-fixing power although their neutralizing titers were low. It may be added, that the results of

‡ All antigens, with the exception of American Q fever, were prepared by benzene extraction procedures. The American Q fever antigen was an ether extracted-washed rickettsial body preparation.

TABLE IV.

Comparison of Complement-fixation and Neutralization Tests with Colorado Tick Fever Human Convalescent Sera.

Serum	Blood* taken	Antigen	Complement-fixation tests† Serum dilutions 1:1	1:2	1:4	1:8	1:16	1:32	Neutralization tests‡ Mortality ratio of mice inoculated with virus dilutions 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	LD_{50} titer	Calculated Protective index§
No. 1	33 mos.	Fl	±	0	0	0	0	0	6/6	6/6	6/6	6/6	4/5	0/5	1/5		$10^{-5.50}$	4
No. 2‖,¶	2½ ''	Fl	4	4	4	4	±	0		6/6	3/5	4/5	0/6	0/5			$10^{-4.10}$	112
No. 3‖,¶	2½ ''	Fl	4	4	4	±	0	0		6/6	5/6	3/6	2/6	0/6			$10^{-4.15}$	100
No. 4‖	42 ''	Fl	±	0	0	0	0	0		6/6	6/6	3/6	0/5	0/5			$10^{-4.00}$	141
No. 5	7 ''	Fl	4	4	4	1	0	0	6/6	3/6	0/5	0/5					$10^{-2.00}$	14,130
No. 6	34 ''	Fl	4	4	4	1	0	0	6/6	4/6	1/6	1/6					$10^{-2.45}$	5,012
Ca	2 days	Fl	2–	0	0	0	0	0			6/6	6/6	5/6	1/6	0/6		$10^{-5.50}$	4
Og	9 ''	Fl	3	2	0	0	0	0			6/6	6/6	0/6	0/6			$10^{-4.50}$	45
Mo	14 ''	Fl		4	4	4	3	±			6/6	5/6	1/6	0/6			$10^{-4.50}$	45
		B		4	4	2	0	0										
		C		4	4	1	0	0										
Wi	18 ''	Fl		4	4	2	0	0			6/6	4/6	2/6	1/6			$10^{-4.60}$	35
		B		4	3	0	0	0										
		C		4	3	0	0	0										
Normal control												6/6	6/6	3/6	0/6	1/6	$10^{-6.15}$	0
Su	2nd febrile rise	Fl		0	0	0	0	0			6/6	6/6	6/6	5/6	0/6		$10^{-6.40}$	0**
	22 days	B		4	4	4	0	0			6/6	6/6	4/6	0/4			$10^{-5.25}$	14**
		C		4	4	4	0	0										
	76 ''	B		4	4	3	0	0		6/6	5/6	1/4	0/6	1/6			$10^{-3.40}$	1,000**
		C		4	4	4	0	0										
		Kolmer		0	0	0	0	0										

* Counting from day when diagnosis of Colorado tick fever was made.
† Complement-fixation tests made by C.J.D. and L.J.K.
‡ Neutralization tests made by H.K.
§ Based on difference in LD_{50} titer from normal control serum.
‖ Induced experimental infection.
¶ Sera No. 2 and No. 3 were anticomplementary in dilutions 1:1 and 1:2 to 2+ and 4+ respectively.
** Based on difference between convalescent and acute phase sera.

complement-fixation on the above sera were in close agreement regardless of whether the Fl, B or C antigens were used in the test.

The results of the tests with Sn sera are perhaps more illustrative than the above cited instances. Serum samples were obtained from the same individual at various intervals during and after illness. It may be observed that the Sn serum drawn during the second febrile rise was free from complement-fixing, as well as neutralizing antibodies. On the other hand, serum taken 22 days later showed positive complement-fixation in 1:8 dilution but neutralized only 14 LD_{50} doses of virus in mice. The complement-fixing antibody titer showed no significant difference between the 22nd and 76th days after diagnosis, but the neutralizing index of the serum increased from 14 to 1,000 during the same period. Again, the results of complement-fixation with the 3 samples of Sn serum were the same regardless of whether B or C antigens were employed in performing the test.

Summary and Conclusions. Specific diagnostic complement-fixing antigens for Colorado tick fever have been prepared from infected mouse brains. The benzene-extracted antigens employed gave no false positive reactions in the presence of highly positive human syphilitic sera. Cross fixation tests between Colorado tick fever immune serum and the heterologous antigens of viral and rickettsial origin indicate that Colorado tick fever is a distinct entity and the virus is not related to any of the other infectious agents tested. These results confirm those obtained with the mouse neutralization test previously reported.[13,14] Close correlation was obtained in the complement-fixation and mouse neutralization tests with human convalescent sera. The complement-fixing and neutralizing antibodies apparently appear in the blood of humans at about the 9th to 14th day after diagnosis of illness and may remain demonstrable as long as 34 months later.

The complement-fixation test, as well as the mouse neutralization test, may prove of value in epidemiological studies on the incidence and geographic distribution of Colorado tick fever. Furthermore, if results with animal sera parallel those obtained with human sera, the tests may be applied to study the ecology of Colorado tick fever.

15747

Studies on Bacterial Resistance to Streptomycin.*

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The rapid development of resistance to streptomycin by various organisms, both *in vitro* and clinically, has recently been reported by a number of workers.[1-4] Miller and Bohnhoff[1] have shown that gonococci and meningococci, originally susceptible to 8-40 units per cc can become resistant to concentrations of streptomycin as high as 75,000 units per cc within 4 to 6 transfers. Klein and Kimmelman[2,3] found that with the 12 strains of Shigella studied, resistance was increased from concentrations of 3-7 units to

* These investigations were supported by grants from Abbott Laboratories, Eli Lilly and Company, Lederle Laboratories, Inc., Parke, Davis and Company, and the Upjohn Company.

1 Miller, C. P., and Bohnhoff, M., *J. A. M. A.*, 1946, **130**, 485.

2 Klein, M., and Kimmelman, L. J., *J. Bact.*, 1946, **51**, 581.

3 Klein, M., and Kimmelman, L. J., *J. Bact.*, 1946, **52**, 471.

4 Alexander, H. E., *J. Pediatrics*, 1946, **29**, 192.

1,000 units of streptomycin per cc, in from 2 to 11 transfers, depending on the particular strain. Clinically, Alexander[4] reported the isolation, from 2 patients, of 2 strains of *H. influenzae* which had the capacity to thrive in the presence of streptomycin in a concentration of 1,000 units per cc. The study reported in the present paper was undertaken to determine: (1) the ease with which resistance to streptomycin could be developed by various organisms; (2) the degree and duration of such resistance; and (3) the ability of resistant strains to grow in freshly defibrinated blood from a normal adult as compared with that of their parent, susceptible strains.

Materials and Methods. The strain of staphylococcus used throughout the tests was a *Staphylococcus aureus* (Merck test strain SM) obtained from Sydenham Hospital.† The strain of *H. influenzae,* type b, was isolated from a patient with influenzal meningitis in Sydenham Hospital. The pneumococcus, strain SVI, was a type I organism originally isolated in 1938 and kept since then on artificial media, with frequent passage through mice to maintain its virulence, until the beginning of the experimental period. The beta hemolytic streptococcus used was a Group A, type 14 strain obtained from Dr. Rebecca Lancefield in 1938. It was received in lyophilized form and was maintained in that state until the beginning of this study. Following initial experiments all cultures were transferred at 3- to 4-week intervals on suitable culture media not containing streptomycin. No attempt was made to maintain the original virulence of the strains during the experimental period.

The susceptibility of the strains to streptomycin was determined by the use of a series of tubes of nutrient broth containing falling concentrations of streptomycin, ranging from 125 to 6.25 μg per cc expressed as pure base.‡ Each tube contained 10% less streptomycin as measured against the initial tube in a regular progression so that the interval 125 to 6.25 represented 15 tubes. Each tube was inoculated with 0.05 cc of an 18-hour broth culture in such dilution as to yield an inoculum of 200,000 to 2 million organisms per tube. The tubes were incubated at 37°C and readings were made at the end of 24 hours. End points by this method were so apparent that plating of the mixtures of organisms and streptomycin after 24 hours incubation was not necessary. When, toward the end of this study, it became necessary to enumerate bacterial colonies, streptomycin dilutions in one cc amounts were added directly to tubes containing 9 cc melted nutrient agar and after thorough mixing were poured into sterile petri dishes. Quantitated inocula of strains to be examined were then streaked on the surfaces of these plates. The number of micrograms of streptomycin in a series of plates varied from 10 to 1000 per cubic centimeter.

After the initial susceptibility of the various test strains to the action of streptomycin had been determined, each strain was transferred daily to broth tubes containing increasing concentrations of streptomycin. To provide adequate growth factors, 20% rabbit serum was added to the broth in which streptococci and pneumococci were grown, 5% Fildes' peptic digest was added to the broth in which the influenza bacilli were grown, but infusion broth alone proved adequate for the growth of the staphylococci. Control cultures containing no streptomycin were also transferred at daily intervals on all test strains.

The bactericidal power of freshly defibrinated human blood on all strains, both before and after the acquisition of resistance, was determined in parallel each time with the same blood specimen. Five hundredths cc of each 10-fold serial dilution (10^{-1} to 10^{-6}) of an 18-hour broth culture was added to 0.25 cc of freshly defibrinated human blood in each of 6 sterile pyrex tubes. These tubes were sealed, placed in a rotating box and maintained at 37°C for 24 hours. After preliminary observation, incubation without rotation was continued for another 24 hours,

† These strains from Sydenham Hospital were obtained through the courtesy of Dr. Horace L. Hodes and Miss Helen Zepp.

‡ The preparation of streptomycin employed had a potency of 1000 units per milligram of pure base. (Pfizer, lot No. 4613.)

after which the tubes were opened and the contents cultured on blood agar plates. In order to determine the number of organisms added to each tube of blood, count plates were made of the 10^{-5} and 10^{-6} dilutions.

Results. (1) when tested initially, the staphylococcus was inhibited by 8 μg of streptomycin per cc; the *H. influenzae* by 1 μg; the pneumococcus by 37 μg; and the streptococcus by 25 μg per cc. After 6 daily transfers in the presence of streptomycin, the pneumococcus and the streptococcus grew in maximal concentrations of 125 μg per cc. The staphylococcus, originally inhibited by 8 μg, was able to multiply in 1,000 μg per cc after 12 daily transfers. The *H. influenzae,* initially inhibited by 1 μg, grew in 60 μg per cc after 14 daily transfers. Unfortunately, at this time both the parent and resistant strains of *H. influenzae* were lost and further studies could not be carried out.

(2) The susceptibility to streptomycin was retested on all strains (parent, parallel control and resistant) after they had been transferred repeatedly in the absence of streptomycin for 6 months. It was found that the parent and control strains of the streptococcus, pneumococcus and staphylococcus were still inhibited by the same concentrations of the antibiotic. The resistant streptococcus, although not in contact with streptomycin for this period, had not only maintained its ability to grow in 125 μg per cc, but actually showed an increase in resistance in that it could now grow in 500 μg per cc. Growth was inhibited at 1,000 μg per cc. The resistant staphylococcus had also maintained its capacity to grow in 1,000 μg per cc and when tested in even higher concentrations, it was found to grow abundantly in 5,000 and 10,000 μg per cc. Moderate growth was observed in the presence of even 50,000 μg per cc. This strain was not tested at higher concentrations of the drug because the supply of streptomycin was still limited. Unlike the streptococcus and the staphylococcus, the resistant pneumococcus did not maintain its ability to grow in 125 μg of streptomycin per cc. When retested, it was found to be inhibited by concentrations greater than 87 μg per cc.

At the time of reexamination of these strains for their susceptibility or resistance to streptomycin one additional set of experiments was initiated. If resistant strains are mutants thrown off by susceptible strains, it should theoretically be possible to select them much more readily in very young cultures. Accordingly, the susceptible staphylococcus was grown in broth at 37°C for 1 hour, 2 hours and 4 hours, after which a known inoculum of each culture was put into broth to which streptomycin had been added in the following final concentrations: 0, 10, 25, 75, 100, 250, 500, 750, 1000 μg per cc. The tubes were incubated at 37°C for 24 hours and the results were then read. All tests were run in duplicate.

It was found that with an inoculum of 200 million organisms per tube the one-hour culture was able to grow in a concentration up to 25 μg of streptomycin. The 4-hour culture, in an inoculum of 11 million organisms per tube (the culture having been diluted to 10^{-2}, because growth was so heavy at 4 hours) grew out in 10 μg of streptomycin per cc but not at higher concentrations. Growth was observed with the 2-hour culture with an inoculum of 400 million bacteria per tube at 10, 25, 50, 100, 500, 750 and 1000 μg of streptomycin per cc. No growth was apparent in those tubes containing 75 μg per cc. Thus, at a very early stage of growth it was possible, by chance selection, to isolate highly resistant mutants. When these resistant mutants were subcultured to broth containing streptomycin it was found further that they bred true in that they grew in the same concentrations or higher. Therefore with a single transfer, resistance to streptomycin in a concentration as high as 1,000 μg per cc was obtained.

An attempt was made to explain this last observation. A series of agar plates containing 0, 10, 25, 50, 75, 100, 250, 500, 750 and 1,000 μg of streptomycin were inoculated with 0.1 cc amounts of a 10^{-5} dilution of each of the following: (1) the original staphylococcus which grew initially in not more than 8 μg of streptomycin; (2) the one-

TABLE I.

Proportion of Organisms in Various Staphylococcus Cultures Growing at Indicated Streptomycin Concentrations.

Test inoculum of staphylococci	Concentration of streptomycin in medium (μg per cc)																			
	0		10		25		50		75		100		250		500		750		1000	
	Colonies		Colonies		Colonies		Colonies		Colonies		Colonies		Colonies		Colonies		Colonies		Colonies	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Control culture	720	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Culture with growth at 25 μg streptomycin per cc	640	100	—	—	200	31.3	2	0.3	2	0.3	4	0.6	2	0.3	0	0	0	0	0	0
Same at 100 μg	1200	100	—	—	—	—	—	—	—	—	800	66.5	920	76.6	800	66.6	760	63.2	720	60
Same at 500 μg	700	100	—	—	—	—	—	—	—	—	—	—	—	—	700	100	600	85.7	600	85.7

hour culture which grew in not more than 25 μg; (3) the 2-hour culture which grew at 100 μg, and (4) the 2-hour culture which was capable of growth in the presence of 500 μg. After 36 hours incubation at 37°C, colonies were observed with the original staphylococcus on the plate containing no streptomycin, but none were present on those plates containing 10 to 1,000 μg. The one-hour culture which grew initially in 25 μg showed abundant growth on plates containing streptomycin up to this level. On each of the plates containing 50, 75, 100 and 250 μg, 2 to 4 colonies grew out, indicating that approximately 1% of the inoculated organisms were resistant to higher concentrations of streptomycin while 99% were inhibited by more than 25 μg per cc. The 2-hour culture, resistant to 100 μg in broth, grew well on all plates. Colony counts indicated that most of the organisms able to grow in 100 μg could also grow in all 4 higher concentrations. Likewise, with the 2-hour culture originally resistant to 500 μg, almost all the organisms were found to grow in concentrations of 500, 750 and 1,000 μg. These results are summarized in Table I.

(3) A comparison of the ability of parent and resistant strains to grow in fresh human blood as in a bactericidal test was the third objective of this study. During the initial period of these experiments, a comparison of the ability of parent, parallel control and resistant strains to grow in normal human blood revealed no difference with the 3 organisms tested—the staphylococcus, the pneumococcus and the streptococcus. As measured by this method, the virulence or potential invasiveness of all 3 resistant strains was unchanged with respect to the virulence of the homologous parent strains.

When retested 6 months later, it was found that the virulence of the parent, control and resistant strains of the pneumococcus had diminished markedly and to the same extent. All 3 strains, parent, control and resistant, of staphylococci had, on the other hand, maintained their original virulence. Finally, in the case of the streptococcus, whereas the parent and control strains showed marked

TABLE II.
Summary of Bactericidal Studies on Streptomycin Susceptible and Resistant Strains.

Organism	Strain examined	Streptomycin resistance, μg per cc	Min. No. of organisms required to initiate growth per cc of blood	
			Initial period	Six-month re-test
Pneumococcus	*Parent	37	160	100,000
	Resistant	125	340	100,000
Staphylococcus	*Parent	8	320	500
	Resistant	1000-50,000	200	150
Streptococcus	*Parent	25	32	120,000
	Resistant	125-500	36	160

* Since the parallel control strains gave essentially identical results as the parent strains in every instance, they were not recorded in this table.

diminutions in virulence, the resistant strain had retained its original virulence. This observation was repeated and no evidence of a decrease in titre was found. These results are summarized in Table II.

Discussion. Streptomycin resistance can readily be induced *in vitro*. In these studies, the relative ease was found to vary with the particular organism used. With the streptococcus, pneumococcus, and *H. influenzae*, the initial resistance could be enhanced from 4 to 20 times by serial passage in streptomycin containing media. The strain of staphylococcus, originally completely inhibited by 8 μg was observed to acquire the ability to grow in the presence of more than 50,000 μg of streptomycin.

Although repeated serial passage appeared necessary to select out the resistant variants, further study with the staphylococcus revealed that in the early logarithmic stage of growth it was possible, by chance selection, to isolate highly resistant mutants in a single transfer.

The proportion of resistant organisms present in an inoculum could be estimated by seeding streptomycin containing plates. The original strain was completely inhibited by 10 μg. A strain found to be resistant to 25 μg per cc contained 33% resistant colonies at this drug level as compared with the control plate. This same strain contained 0.2% to 0.4% colonies resistant to 50, 75, 100 and 250 μg of streptomycin per cc. The proportion of colonies resistant to higher concentrations up to 1,000 μg of streptomycin was exceedingly high (60 to 100%) when the inoculum was derived from strains found resistant to 100 μg or greater. This distribution of resistant colonies was present when serial transfer or random selection from young cultures was used as the screening procedure. In the usual method for determining streptomycin resistance, the small number of resistant organisms present is probably insufficient to initiate growth and, therefore, it may not contribute to the observed end point. The rate at which resistance is apparently acquired is a function of the proportion of resistant organisms present at each successive level. This phenomenon may also explain erratic results sometimes noted. The growth which appears at a high level of streptomycin despite the absence of growth at lower levels, may be attributed to the random selection of resistant variants.

Repeated transfer by subculture on non-streptomycin containing media over a period of 6 months did not result in a loss of streptomycin resistance, with the possible exception of one strain, the pneumococcus.

The bactericidal studies were performed as an index of virulence. No loss in potential invasiveness accompanied the acquisition of streptomycin resistance. After serial transfer of parent, parallel control and resistant strains for a period of 6 months, the ability of the pneumococci to survive in whole blood was greatly diminished while that of the staphylococci was unchanged. With the susceptible parent and parallel control strains of streptococci a marked diminution was noted, but the resistant streptococcus retained its potential invasiveness.

The general experience has been that strains which become resistant to penicillin lose their invasive properties as indicated by growth in whole blood.[5,6] Strains which have developed resistance to sulfonamides, however, retain this capacity unchanged.[7] From the present observations it appears that, like the latter, organisms resistant to streptomycin may also retain their virulence. In addition, such strains may preserve the ability to maintain their invasiveness upon prolonged artificial cultivation to a greater extent than the parent, nonresistant culture. The clinical implications of these observations that invasiveness may be maintained and retained are evident. The development of streptomycin resistance should be reduced to a minimum through the use of a dosage schedule which will ensure early, adequate blood levels. In the light of these studies, an adequate level must be defined in terms of the specific susceptibility of the organism plus its potential ability to acquire resistance. The level of tolerance to streptomycin as well as the rate at which this tolerance is acquired are important factors and a large margin of safety is necessary.

Summary. 1. Resistance to streptomycin can be induced by repeated transfer of various organisms in streptomycin containing media. 2. In the early logarithmic stage of growth, highly resistant mutants can be isolated, by chance selection, in a single transfer. 3. In most instances, acquired resistance to streptomycin is maintained. 4. Organisms resistant to streptomycin may retain their original virulence as measured by the bactericidal test. 5. The acquisition of resistance to streptomycin with the maintenance of virulence may have certain therapeutic implications.

5 Spink, W. W., Ferris, V., and Vivino, J. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 210.

6 Rake, G., McKee, C. M., Hambre, D. M., and Houck, C. L., *J. Immunol.*, 1944, **48**, 271.

7 Chandler, C. A., and Janeway, C. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 179.

15748

Effect of Atabrine on Auricular Fibrillation in the Dog.

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At present there are no available data concerning the action of atabrine on cardiac muscle. However, the analogous physiological effects of quinine and atabrine in diminishing the contractibility and increasing the refractory period of striated and smooth musculature,[1] lead to a reasonable assumption that atabrine will produce similar actions to those of quinine on cardiac muscle.

Quinine and especially its more powerful isomer, quinidine, have been used for many years as a routine practice in the treatment of auricular fibrillation presumably because of their ability to prolong the refractory period in cardiac muscle and its nodal tissues as well as to decrease myocardial excitability.[2] Since recent evidence has been advanced[1,3] which clearly indicates that atabrine possesses more potent physiological and pharmacological properties when compared with quinine, it seemed most interesting to see whether atabrine would also control auricular fibrillation. Experiments in the dog were, therefore, devised to test the validity of this concept.

Methods. Five dogs were anesthetized

1 Keogh, P., and Shaw, F. H., *Australian J. Exp. Biol. and Med. Sci.*, 1944, **22**, 139.

2 Lewis, T., Drury, A. N., Iliescu, C. C., and Wedd, A. M., *Heart*, 1921-22, **9**, 207.

3 Gertler, M. M., and Karp, D., *Revue Can. de Biol.*, in press.

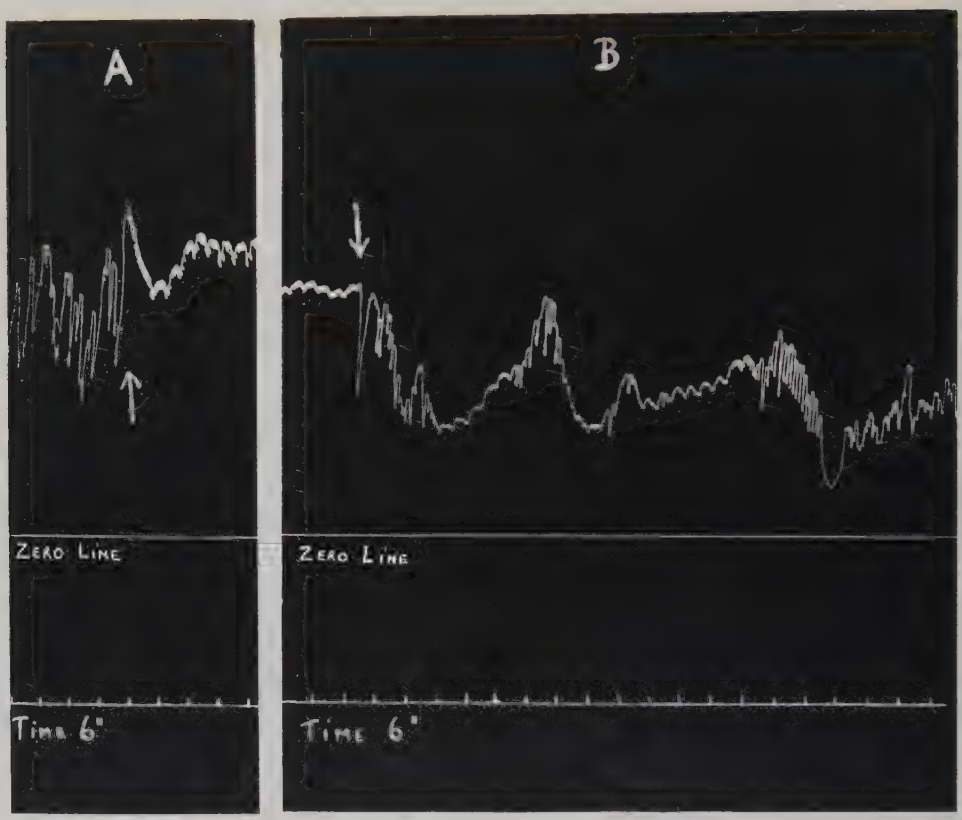

FIG. 1.

A. Restoration of normal sinus rhythm to auricular fibrillation 3 min. 24 sec. after intravenous injection of atabrine was started (Exp. 1).

B. Induction of auricular fibrillation approximately 2 hours after injection of atabrine.

with nembutal (25 mg per kg). Auricular fibrillation was produced according to the method of Hoff and Nahum.[4] Application of mecholyl (0.2% solution in normal saline) over the sino-auricular node, followed by stimulation of any region on the right auricle with a weak faradic current consistently provoked auricular fibrillation. Auricular fibrillation produced in this manner persists for at least 10 to 15 minutes and often longer. Attempts to reproduce auricular fibrillation were made immediately after its restoration to normal sinus rhythm by atabrine. One animal received 1.0 mg of eserine (physostigmine sulphate) intravenously before such an attempt was made.

A Sanborn cardiette was employed for electrocardiographic recordings. Lead II was used throughout the experiments with the animals placed in the dorsal recumbent position.

Atabrine dihydrochloride (Winthrop Chemical Co.), a 0.5% solution in normal saline, was always freshly prepared for these experiments.

Electrocardiographic and blood pressure records were taken immediately prior to and during auricular fibrillation. These records were continued during the infusion of atabrine and continued thereafter until the heart returned to its normal rhythm.

Experimental Results. The restoration of regular sinus rhythm to auricular fibrillation occurred rapidly in all the 5 animals following the intravenous infusion of atabrine (Table I). The gradual recovery to normal rhythm in Experiment 1 can be seen in the

[4] Hoff, H. E., and Nahum, L. H., *Am. J. Physiol.*, 1940, **129**, 428.

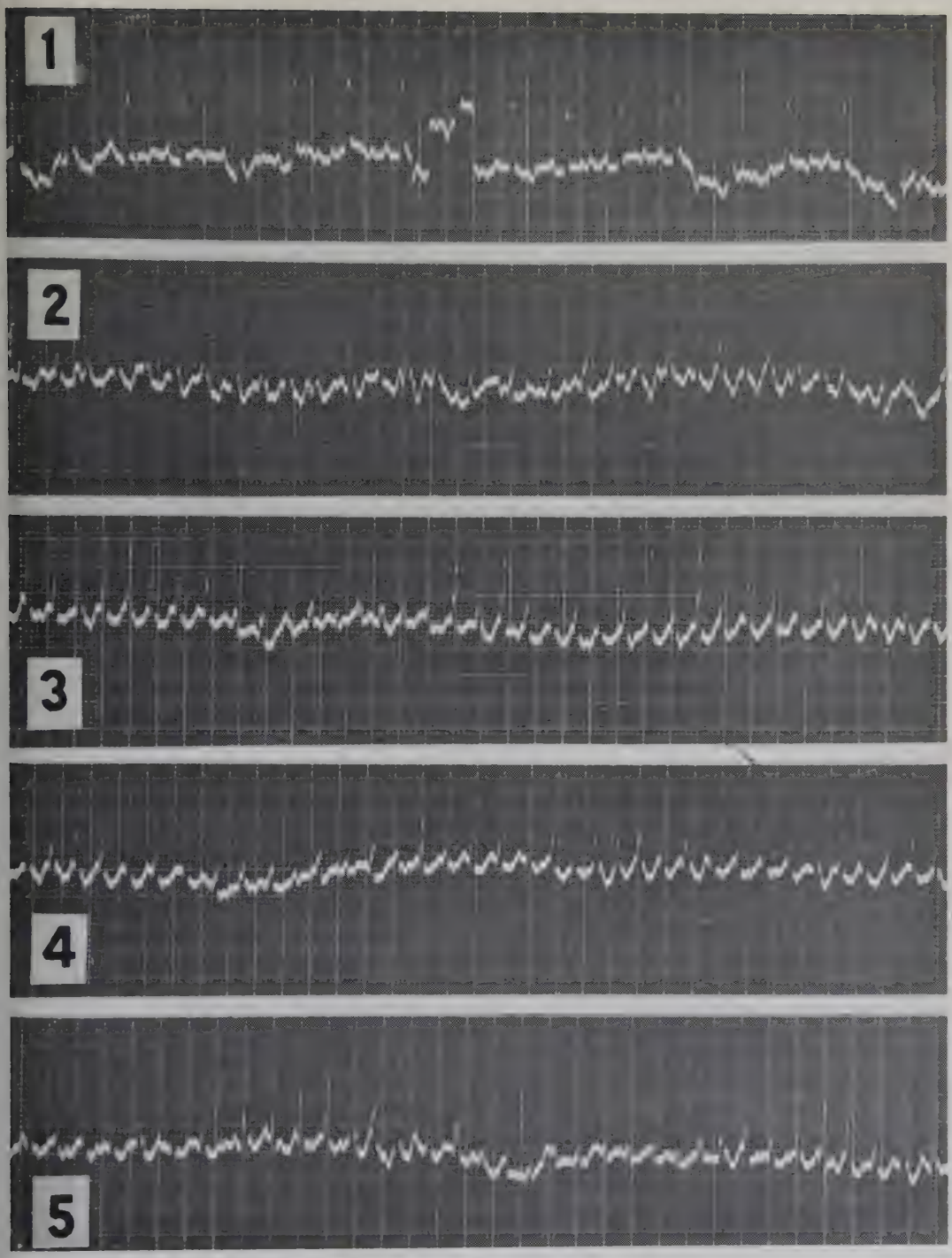

FIG. 2.

Electrocardiogram. Lead II. Same experiment as in Fig. 1. 1—Control record. Heart rate 150 at 10:30 a.m. 2—Auricular fibrillation. Heart rate 234 to 312 at 10:35 a.m. 3—Infusion of atabrine started. 4—5 mg atabrine injected. 5—5 mg atabrine injected.

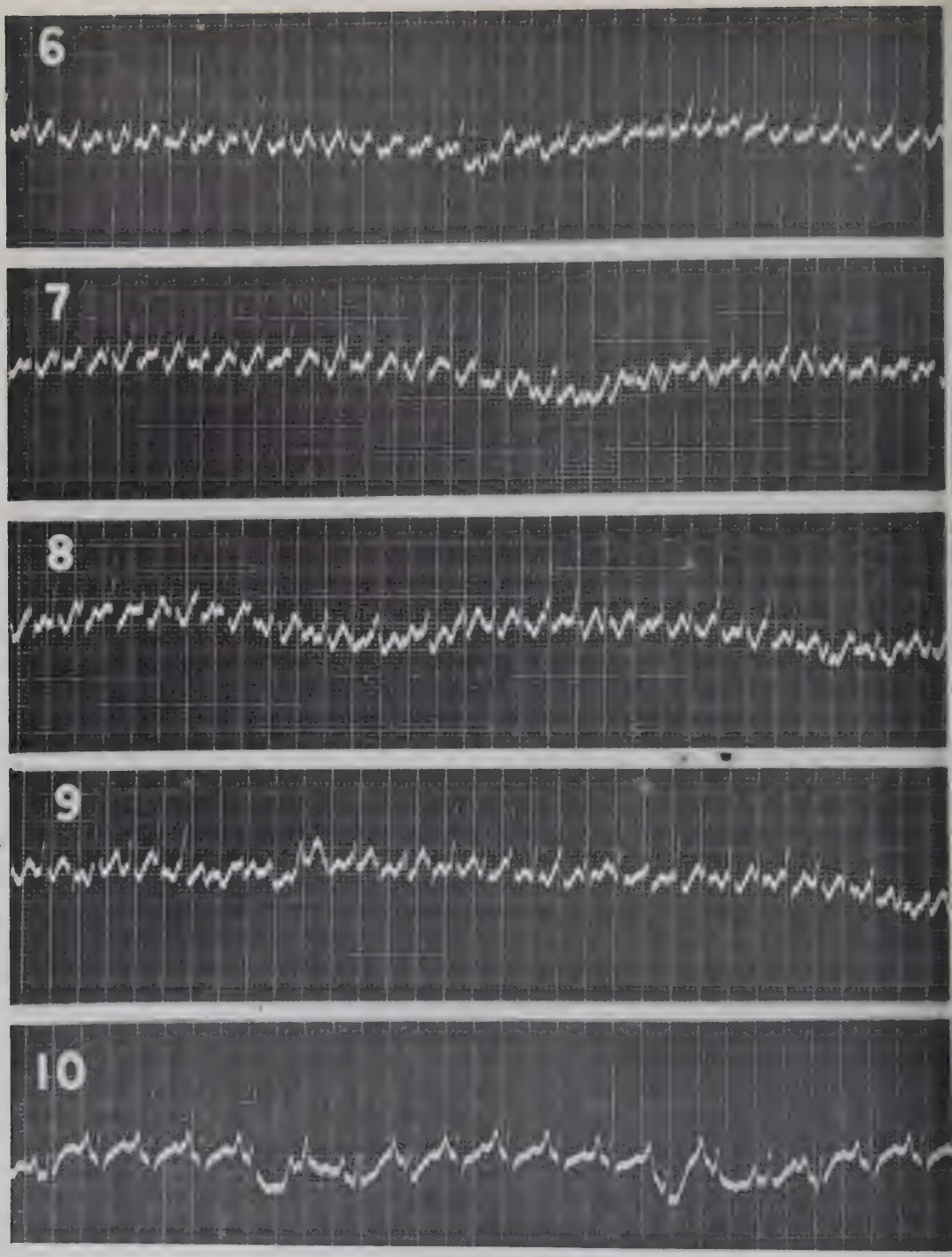

FIG. 3.

Electrocardiogram. Lead II. Continuation of Fig. 2. 6—5 mg atabrine injected. 7—5 mg atabrine injected. 8—5 mg atabrine injected. 9—7.5 mg atabrine injected. 10—Return to normal rhythm after total injection of 37.5 mg. Heart rate 167 at 10:40 a.m. See Fig. 1A.

TABLE I.

Exp. No.	Wt of dog (kg)	Total amt of atabrine (mg)	Mg/kg
1	15	30.0	2.0
2	26	37.5	1.44
3	13.6	40.0	2.94
4	12.5	33.5	2.65
5	8	22.5	2.81

kymographic and electrocardiographic records (Fig. 1, 2 and 3).

Several phenomena were observed in the ventricles. During the early period of atabrine injection the ventricles contracted with great irregularity which disappeared as the concentration of atabrine in the circulating blood was increased by virtue of the continuous infusion. As the infusion of atabrine was continued, the usual accompanying variations in the amplitude of the R wave became less pronounced despite the persistent auricular fibrillation (Fig. 2 and 3). It was also observed that intracardiac injection of atabrine failed to restore the fibrillating ventricle in 2 of the experiments.

The effect of atabrine was long-lasting. Attempts to reproduce auricular fibrillation after its arrest by atabrine were unsuccessful for approximately 2 hours in 2 experiments. In the remaining 3 experiments where auricular fibrillation was reproduced successfully, it was necessary to combine the application of mecholyl pledgets in the region of the S-A node with faradic stimulation of the right auricle for at least 30 seconds. The auricular fibrillation produced by this method was transient, rarely exceeding more than 20 seconds.

Discussion. Experimental evidence for another possible clinical application of atabrine has been brought out in the study of atabrine on the dog heart. The need for a drug in the treatment of auricular fibrillation which is as effective as digitalis or quinidine and which is not as toxic is obvious. It is noteworthy that atabrine, a drug which is efficacious in malaria therapy and which has proven itself to be virtually harmless by its long clinical record, should also control auricular fibrillation. After a survey of the literature, the only reference to atabrine and auricular fibrillation found was that made by Ganguli.[5] While studying the effects of atabrine on the electrocardiogram, in patients with malaria, he included one case which suffered from auricular fibrillation concomitant with malaria. The final electrocardiographic record did not reveal auricular fibrillation, but whether this may be attributed to atabrine or to the conventional forms of therapy cannot be stated with certainty from his article.

Obviously, any drug which reconverts auricular fibrillation into normal sinus rhythm must of necessity increase the refractory period of the "circus movements" in the auricular muscle and diminish the rate of conduction in these circuits.[4] No attempt was made in these experiments to analyze the mechanism by which atabrine restored regular sinus rhythm to auricular fibrillation. The auricular fibrillation produced in these experiments was of the vagal type, *viz.* increased vagal tone plus superimposed auricular excitation. It would be reasonable, therefore, to explain the action of atabrine in restoring regular sinus rhythm to this type of auricular fibrillation on the basis that atabrine paralyzes the vagal inhibitory fibers to the dog heart.[3] However, this explanation is not entirely adequate, for it is known that atabrine possesses such properties as decreasing the excitability of excised auricular tissue,[6] perfused hearts[7] and increasing the refractory period in skeletal muscle.[1]

In order to test the validity of whether the beneficial effect of atabrine in auricular fibrillation was merely due to its antivagal action, it was decided to inject eserine into an animal immediately after atabrine had restored regular sinus rhythm to the experimentally produced auricular fibrillation and observe whether it was possible to reproduce the fibrillation. It was found that auricular fibrillation could be produced with great difficulty and persisted for only 10 seconds. Furthermore, an exclusive antivagal effect is

5 Ganguli, P., *Arch. f. Schiffs-u-Trop. Hyg.*, 1933, **37**, 413.

6 Suffolk and Berkshire, Earl of, *Quart. J. Exp. Physiol.*, 1939, **29**, 1.

7 Chin, K., *Japan J. Med. Sci., IV, Pharm.*, 1937, **10**, 162P.

opposed by the fact that it was impossible to reproduce auricular fibrillation in 2 experiments for a long period after its reconversion into normal sinus rhythm by atabrine despite the presence of a normal electrocardiogram.

While there is no experimental proof as yet, it is reasonable to assume from the evidence submitted that the mechanism of the action of atabrine on auricular fibrillation is similar to that of quinine and quinidine.

Summary. 1. Experimentally produced auricular fibrillation in the dog was successfully restored to normal sinus rhythm by the intravenous infusion of atabrine (average 2.17 mg per kg). 2. The mechanism by which atabrine might produce this effect is discussed. It is suggested that its action is similar to that of quinine or quinidine.

The authors are indebted to Dr. B. P. Babkin for suggesting this problem and for his advice and unfailing interest. To Dr. H. E. Hoff the authors are grateful for his valuable advice and criticism.

This work was supported by a grant to one of us (D. K.) from the Banting Research Foundation.

15749 P

Effect of Penicillin on Growth and Toxin Production of Enterotoxigenic Staphylococci.*

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Little is known of the effect of penicillin on formation or activity of bacterial toxins. Ercoli, Lewis and Moench[1] demonstrated *in vitro* neutralization of diphtheria toxin-antitoxin mixtures; Boor and Miller[2,3] obtained protection *in vivo* against meningococcal and gonococcal endotoxin and Mason[4] demonstrated inhibition of plasma coagulation by enterotoxigenic staphylococci. Neter[5] failed to obtain neutralization of tetanus toxin and Blair, Carr and Buchman[6] found no inhibition of plasma coagulation and formation of hemolysin by staphylococci.

The present paper reports experiments on the effect of penicillin on formation of staphylococcal enterotoxin, hemolysins, lethal factor and dermonecrotoxin.

Tested by the tube dilution method,[7] the natural resistance of 15 enterotoxigenic strains of staphylococci was found to range from 0.05 units/ml to 500 units/ml of penicillin. Three enterotoxigenic (No. 147, 161, 196) and one nonenterotoxigenic (No. 43) strains used in the following experiments were naturally resistant to 500, 30, 100 and 0.1 units/ml of penicillin respectively. The resistance of strains 161 and 43 was further increased to 2000 and 10 units/ml respectively by growth in increasing penicillin concentrations. All strains were alpha hemolytic and strain 196 produced potent beta lysin.

Toxin production in the presence and absence of penicillin was tested in semisolid (0.7%) veal infusion agar and in liquid and semisolid media containing 1% Amigen,† 0.25% glucose, 1.2 μg/ml nicotinic acid, 0.05

* This work was supported in part by a grant from the National Canners Association.

1 Ercoli, N., Lewis, M. N., and Moench, L. J., *J. Pharm. and Exp. Therap.*, 1945, **84**, 120.

2 Boor, A. K., and Miller, C. P., *Science*, 1945, **102**, 427.

3 Miller, C. P., and Boor, A. K., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 18.

4 Mason, H. C., *J. Immunol., Virus Res. and Exp. Chemother.*, 1945, **51**, 307.

5 Neter, E., *J. Infect. Dis.*, 1945, **76**, 20.

6 Blair, J. E., Carr, M., and Buchman, J., *J. Immunol., Virus Res. and Exp. Chemother.*, 1946, **52**, 281.

7 Kolmer, J. A., *Penicillin Therapy*, D. Appleton-Century Co., Inc., New York, N.Y., 1945

† Pancreatic hydrolysate of casein free of vitamins and carbohydrate. Product of Mead, Johnson and Co.

TABLE I.
Effect of Penicillin on the Production of Hemolysins, Lethal Factor and Dermonecrotoxin by Staphylococci.

Toxic factor	Staphylococcus strains*	Medium: Amigen liquid, Penicillin	Amigen liquid, No penicillin	Amigen agar, Penicillin	Amigen agar, No penicillin	Veal infusion agar, Penicillin	Veal infusion agar, No penicillin
Rabbit cell hemolysin (MHD/ml)†	161 N		64		128		
	161 R	<2	32-64	64	64	8	64
	147 N	<2	32-64	128	128	128	128
	196 N	<2	32-64	128	128	64	256
	43 N		32		64		128
	43 R	4	16	16	8	32	64
Sheep cell hemolysin (MHD/ml)†	161 N		<2		0		
	161 R	0	0	8	0	0	8
	147 N	0	0	<2	<2	0	0
	196 N	<2	32	32	128	64	512
	43 N		0		8		
	43 R	0	0	0	0		
Lethal factor	161 N		4/4 (1)		4/4 (.5)		4/4 (.5)
	161 R	0/4 (1.0)‡	4/4 (1)	0/4 (1.0)	4/4 (.5)	4/4 (.5)	4/4 (.5)
	147 N	0/4 (1.0)	4/4 (1)	4/4 (0.5)	4/4 (.5)	4/4 (.5)	4/4 (.5)
	196 N	0/4 (1.0)	4/4 (1)	4/4 (0.5)	4/4 (.5)	4/4 (.5)	4/4 (.5)
	43 N		4/4 (1)		4/4 (.5)		4/4 (.5)
	43 R	0/4 (1.0)	3/4 (1)	4/4 (1.0)	4/4 (1.0)	4/4 (.5)	4/4 (.5)
Dermonecrotoxin	161 N		+§				
	161 R	0	+				
	147 N	±	+				
	196 N	0	+				
	43 N		+				
	43 R	0	±				

* N = normal; R = artificially resistant strains. Strains, except No. 43, are enterotoxigenic.

† MHD (Minimal Hemolytic Dose) of hemolysin indicates least amount of toxin which lyses completely 1.0 ml of a 1% suspension of erythrocytes after incubation for 1 hour at 37°C (rabbit r.b.c.) followed by overnight refrigeration (sheep r.b.c.).

‡ Numerator—number of mice killed within 48 hours; denominator—number mice injected; parenthetic figures—dosage in ml.

§ + = area of necrosis and redness 5 mm or more in diameter; ± = area of redness <5 mm; 0 = no reaction.

μg/ml thiamine HCl, 1.0 μg/ml calcium pantothenate and inorganic salts.[8] All media were adjusted to pH 7.6. Cultures were incubated at 37°C for 3 days. Strains 161 R, 147 N, 196 N and 43 R were grown in 1000, 200, 50 and 5 units/ml of penicillin respectively. Toxins were produced and assays of enterotoxin and hemolysin were carried out by previously described methods.[9-11] Lethal factor was detected by the intravenous injection of mice; dermonecrotoxin by intracutaneous injection of rabbits with 0.1 ml of a 1:20 dilution of toxin in saline.

Counts of viable bacteria revealed no difference in the amount of growth in media containing penicillin compared to the same media without penicillin.

Positive tests for enterotoxin[9,10] were obtained in monkeys or cats with centrifugates of enterotoxic cultures grown in all of the above media with and without penicillin.

Results of assays for hemolysins, lethal factor and dermonecrotoxin are summarized in Table I. The potency of the hemolysins, lethal factor and dermonecrotoxin produced by strains naturally or artificially resistant to penicillin was markedly reduced in cultures grown in Amigen liquid containing penicillin. However, in semisolid Amigen and veal infusion agar cultures, questionable effect of penicillin was noted. Penicillin in the same concentration failed to neutralize preformed hemolysins, lethal factor and dermonecrotoxin.

Summary. The natural resistance of 15 enterotoxic strains of staphylococci to penicillin was found to range from 0.05 to 500 units per ml. Production of staphylococcal hemolysins, lethal factor and dermonecrotoxin, but not of enterotoxin, was inhibited by growing selected naturally and artificially resistant strains in Amigen liquid medium containing sublethal concentrations of penicillin. Inhibition was not observed in similar experiments using semisolid Amigen and veal infusion media.

[8] Surgalla, M. J., and Hite, K. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 244.

[9] Davison, E., Dack, G. M., and Cary, W. E., *J. Infect. Dis.*, 1938, **62**, 219.

[10] Dack, G. M., *Food Poisoning*, University of Chicago Press, 1943.

[11] Surgalla, M. J., and Hite, K. E., *J. Infect. Dis.*, 1945, **76**, 78.

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Studies on Thermal Sedation in Suppression of the Symptoms of Tetanus Toxin

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The following experiments on the physiological effects of temperature suggest that cold narcosis, as seen in reptiles and some warm-blooded vertebrates, will modify the development of symptoms resulting from tetanus toxin. It is possible that cold narcosis might be used as a method of sedation and as an adjunct to therapy in such diseases as rabies and tetanus.

Because of the wide range in temperature tolerance exhibited by the diurnal reptiles and because of their similarity to the mammals with respect to optimum temperature, which in many lizards range from 36°C to 41°C, and because of their thermal simplicity and the greater facility with which these animals can be utilized in temperature experiments, it was agreed that any common diurnal species of reptiles would be suitable for experimentation, and the northern crested lizard *Dipsosaurus dorsalis dorsalis* was selected as being most convenient as to size,

thermal requirements, responses, and availability.

Although it is stated in the literature[1] that reptiles do not respond to tetanus toxin, experience in the field and laboratory suggested that there was great possibility of error in the previous experiment due to lack of appreciation of the vital necessity of maintaining optimum thermal condition. In most experimental work with reptiles, the full importance of the temperature factor has not been recognized.

In the pilot experiment, because reptiles had been reported as not susceptible to tetanus toxin, the arbitrary enormous dose of 20,000 guinea pig M.L.D.'s (minimal lethal dose) was employed in testing a lizard weighing 65 g. It was planned that, in case the symptoms of tetanus did not appear within a few days' time, the animals were to be exposed to optimum temperatures of 34°C to 40°C in the hope that during the approach towards or into this normal temperature range, the onset of symptoms might reveal a critical threshold at which neural and muscular effects might develop and below which partial cold narcosis might prevent their appearance.

Contrary to expectation, the original and all subsequent experiments demonstrated the fact that these animals, when held at temperatures ranging from 10°C through to the optimum of 38°C, are by no means immune to the effects of tetanus toxin although at all low temperatures there is a marked retardation in the appearance of symptoms.

Exp. I. Three specimens of *Dipsosaurus dorsalis dorsalis* were given the following doses: 20,000 guinea pig M.L.D.'s in one instance, for the other 2, 10,000 guinea pig M.L.D.'s each. The toxin was injected intramuscularly in the femoral regions in these and all subsequent experiments. The animals were then maintained at temperatures of 24°C to 26°C until the onset of symptoms. In all instances first symptoms appeared as slight tremors in the head and tail regions. These tremors appeared first in the individual receiving 20,000 guinea pig M.L.D.'s. The first perceptible tremors occurred 36½ hours after inoculation. Two hours after onset of primary symptoms, action of the hind legs was impaired and the tail was arched strongly in a lateral position.

This specimen which had received 20,000 guinea pig M.L.D.'s was heated to 32°C at which time opisthotonic contractions arched the tail and back to a dorsolateral position. Sound or sudden movements taking place in close proximity to the animal caused convulsions, although fairly periodic spasms were observed even without extra stimulation. Three hours after the appearance of symptoms this individual was placed in an incubator refrigerator at a temperature of 10°C-11°C and was maintained under these conditions until death occurred 212 hours later.

The remaining 2 subjects which had been given 10,000 guinea pig M.L.D.'s, only half the dosage administered to the first lizard, showed these initial tremors 40 hours after injection. One of these was immediately placed in a temperature of 10°C-11°C while the other was maintained at room temperature. At room temperature (27°C-28°C) death took place between 72 and 80 hours after inoculation. Prior to death, violent symptoms of tetanic convulsions and opisthotonic contraction threw the animal into a strong sigmoid curve with the head inclined upward at a sharp angle. This posture is retained with only slight relaxation even after death. (Fig. 2).

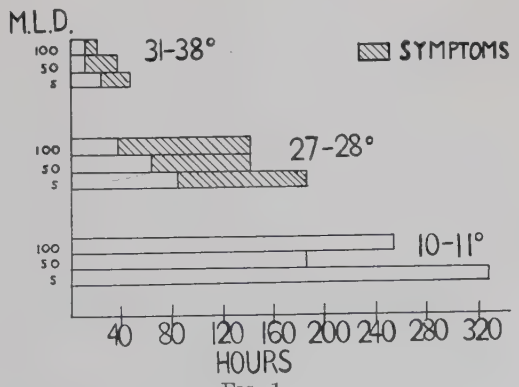

FIG. 1.

Duration of life in desert iguana, injected with tetanus toxin, with respect to maintenance temperature.

[1] Metchnikoff, E., *Ann. Inst. Past.*, 1897, **11**, 801.

FIG. 2.
Dead *Dipsosaurus* showing characteristic posture attendant on death from tetanus.

Eighty hours after inoculation, the third specimen, which had been maintained at 10°C, was removed from the cold chamber and allowed to warm to room temperature, in this case 25.5 to 27°C. On warming, locomotory movements were limited to forelimbs but movement was poorly coordinated. The hind legs were immobilized and were strongly adpressed to the tail, although locomotion could be carried on with the front legs alone.

As temperatures rose from 25.5-27°C, convulsive, tetanic contractions became progressively more violent and, as reported above, it was noticeable that auditory and visual stimuli, as well as vibrations and movements taking place in close proximity to the animal precipitated these convulsive reactions.

At 28°C the body became still more violently flexed, remaining in this position almost continuously. The flexure was so violent that the animal was forced to lie on one side. At 30°C the head was flexed strongly to the left and the forelimbs showed limited mobility and were flexed backwards under the body. After being exposed to warming temperatures for 23 minutes, the animal defecated for the first time and the body temperature was noted as being 31°C. Twenty-five minutes after removal from the cold chamber and while still with body temperature of 31°C, it was first noted that respiratory movement had ceased, but it is possible that respiration may have stopped shortly before.

Concomitant with cessation of respiration, the general reactions became fewer and less severe and the animal remained in a contorted, prostrate position. Only 52 minutes after being removed from the control chamber, and with body temperature having risen to only 31.5°C, the animal died.

Animal No. I, which received double the dosage given the other 2 individuals, was maintained continuously at a temperature of 10°C thus acting as a control for No. II and III. In spite of such a heavy dosage, this animal outlived the others by a total of 212 hours, whereas, the individual chilled for 80 hours then warmed to 32°C died after only 52 minutes at a moderate temperature for these animals. Since the optimum temperature for this species approximates 37°C and may rise to 43°C with no discomfort to the animal, it is clearly apparent that death in so short a time cannot be attributed to overheating or overstimulation. At least one factor causing death from tetanus toxin seems to be the violence of contraction, thus with approach toward normal neuromuscular reactions, the toxin-produced reaction may cause traumatic injury and thus death. It seems improbable that either the amount or the degree of diffusion of the neurotoxic element can have caused death where no symptoms were evoked; on the contrary, this would appear to be a case of death induced by some other toxic effect or by too prolonged chilling, which seems rather improbable, or some other unknown factor.

In these experiments the most clearly discernible effect of temperature is the very marked lowering of the intensity of all tetanic reactions and their gradual intensification attendant on rising temperatures. This cor-

relation of violence of reaction with temperature change is in harmony with the anticipated results and is also in accord with the generally known effect of temperature changes with respect to metabolic rates in general.

Exp. II. The extreme sensitivity of these reptiles to tetanus toxin suggested that a second series of lizards be tested with lighter dosages in order that some approximation to their M.L.D.'s might be obtained prior to any additional expenditure of living material. For this reason, similar treatment was utilized but dosages of 100, 800 and 4,000 guinea pig M.L.D.'s were administered to the animals.

Following inoculation, the lizards were first maintained at 26-28°C; that is, 10° below their optimum. Thus they were held under conditions which seemingly would retard slightly the onset of symptoms.

Although all 3 lizards appeared normal for the first 48 hours, that with 4,000 units showed extreme symptoms 75 hours after inoculation. The 800 guinea pig M.L.D. individual displayed only the primary evidence of involvement, tremors of the head and cervical regions. All 3 animals were placed in the refrigerator incubator at 10°C as soon as symptoms were detected. The 100-unit individual showed no symptoms and was continued at room temperatures pending development of evidence of toxic effect.

After 90 hours at 26-28°C, the individual with 100 guinea pig M.L.D.'s was found to be suffering from partial paralysis of the hind legs and was then placed with the other 2 individuals at a temperature of 10°C.

Death of the 4,000-unit individual occurred sometime between 196 to 214 hours after inoculation, the time lapse probably being somewhat curtailed owing to a 2-hour period of warming to 31°C while photographs were being taken to illustrate symptoms.

The remaining 2 individuals, those at 800 and 100 units, while still refrigerated, showed marked opisthotonic contractions up to 244 hours, these symptoms gradually diminishing in intensity until immobility, possibly from prolonged exposure to abnormally low temperature, concealed the reaction. These conditions led imperceptibly to death some 270 hours ($\pm$ 12 hours) after inoculation.

Exp. III. In this investigation, an attempt was made to accomplish the following:

1. Test more fully the retarding effects of low temperature.

2. Test the accelerating effect of optimum temperature.

3. Determine the effect of small dosages of the toxin on:

   a. Animals continuously cooled to torpidity (10°C) from the time of inoculation to the natural termination of the experiment.

   b. A series maintained at temperatures of 27-28°C, and

   c. A series at temperatures slightly above the optimum but within voluntary tolerance range.

4. Determine the approximate lizard M.L.D. for this species under 3 conditions: namely,

   a. When cooled to torpidity.

   b. Maintained at approximately minimum-activity level, and

   c. At and slightly above the optimum.

Procedure. Nine lizards were divided into 3 groups, one of which received 100 M.L.D.'s, another 50 guinea pig M.L.D.'s, and the third 5 guinea pig M.L.D.'s, thus reducing the dosage from that of the maximum administered in the first experiment to 1/200, 1/400, and 1/4,000 of that massive inoculation. From each of these dosage groups, one individual was placed in the incubator-refrigerator at 10°C, one in a cage at room temperature 26-28°C, and a third was placed in a heating cabinet at 38°C, the approximate optimum temperature, although still 5°C below temperatures selected by these animals under natural conditions and some 7-9° below the lethal temperature for the species. Throughout the first 17 hours in a heating cabinet, temperatures were raised slowly from 31-38°C and thereafter were maintained at the latter level.

In order to postulate the exact relationship between different thermal levels and the progress of the intoxication, larger series of animals as well as greater standardization of

procedures would have been required, conditions not compatible with objectives in these preliminary experiments.

In spite of the exploratory nature of these tentative trials, it is apparent that the life expectancy of these lizards, when under normal thermal conditions—that is, 31-38°C—is less than 2 days; whereas with moderate cooling to 27-28°C or chilling to 10-11°C, there is a material prolongation of life, in the former case by 7 to 8 days, and in the latter as much as 14 days. Unfortunately, controls were not run to determine how long a normal lizard would survive at this temperature (ice box).

Although these results might have been predicted by the use of Van't Hoffs Law, the ameliorating effects are greater than the Q_{10} of 2, and approach those of 3 to 4. This larger factor may be due to inherent differences of tissues or to physiological functions, but at present it seems more probable that the explanation may lie in the realm of normal differences of the Q_{10} for different thermal levels.

Conclusions. 1. Contrary to reports indicating that "cold blooded" creatures are not susceptible to tetanus toxin, the desert iguana is susceptible and for temperature studies is an excellent experimental animal. 2. Lowering of temperature markedly prolongs life in the desert iguana inoculated with tetanus toxin. From Van't Hoffs Law one would expect a slowing down of the effects of the toxin at lower temperatures. 3. There is a tremendous field awaiting study on the effects of altering bodily temperature on the course of disease, especially where toxic factors are concerned.

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Use of Thiosulfate Clearance As a Measure of Glomerular Filtration Rate in Acidotic Dogs.*

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The renal clearance of thiosulfate has recently been proposed as a measure of glomerular filtration rate in the normal dog[1] and in man.[2] Since the majority of the ionic constituents of the glomerular filtrate are more or less completely reabsorbed during their passage through the renal tubules, the apparent tubular rejection of this anion constitutes an interesting anomaly. The present study of the excretion of thiosulfate in the acidotic dog was undertaken with the following 2 ends in view. If thiosulfate were to undergo no reabsorption in the tubules of animals with disturbed acid base relationships, as is apparently true in normal animals, that fact would strengthen the concept that its clearance constitutes an adequate and broadly applicable measure of filtration rate. If the thiosulfate clearance could be used as a measure of filtration rate in acidotic animals, it would have the following advantage over the creatinine clearance. Thiosulfate, unlike creatinine, is devoid of buffer properties. Hence, it can be predicted that the administration of this substance would not affect the rate of excretion of titratable acid, as does the administration of creatinine.[3]

In this study it has been found: (1) that the thiosulfate clearance provides an accurate means of quantitating glomerular function in

* Aided by grants from the John and Mary R. Markle Foundation and from the United States Public Health Service.

1 Gilman, A., Philips, F. S., and Koelle, E., *Am. J. Physiol.*, 1946, **146**, 348.

2 Newman, E. V., Gilman, A., and Philips, F. S., *Bull. Johns Hopkins Hosp.*, 1946, **79**, 229.

3 Pitts, R. F., and Alexander, R. S., *Am. J. Physiol.*, 1945, **144**, 239.

acidotic dogs; (2) that the administration of thiosulfate *per se* does not significantly disturb either glomerular filtration rate or renal plasma flow; and (3) that the administration of thiosulfate does not greatly alter the rate of excretion of titratable acid. Accordingly, the thiosulfate clearance is to be preferred to the creatinine clearance in studies on renal function in animals in acidosis.

Methods. A total of 12 experiments were performed on 3 trained female dogs rendered acidotic by the oral administration of ammonium chloride. Solutions containing creatinine, sodium thiosulfate and sodium *p*-aminohippurate were infused at a constant rate into the saphenous vein. Except in Experiments 1 and 2 the rate of infusion was maintained at 3 cc per minute. In these initial experiments infusion rates of 10 cc per minute gave evidence of expansion of circulating blood volume and increased filtration rate. High infusion rates were therefore discontinued. Plasma and urine thiosulfate concentrations were determined by the plasma method of Newman *et al.*[2] Corrections were made for both plasma and urine blanks. Other procedures and chemical methods have been described in previous communications.[3,4]

Identity of the creatinine and thiosulfate clearances in acidotic dogs. In 50 comparisons of simultaneously determined thiosulfate and creatinine clearances in 3 acidotic dogs, the mean thiosulfate/creatinine clearance ratio was 1.004. In 10 experiments the ratios varied within limits of 0.95 and 1.05. In 2 experiments ratios as low as 0.90 and as high at 1.13 were observed. It is evident within limits of experimental error that the thiosulfate and creatinine clearances are equal in acidotic dogs as they are in normal dogs.[1]

In Table I are presented 2 illustrative experiments in which the plasma concentration of thiosulfate was varied from 17.8 to 55.6 mg %. The plasma concentration of creatinine was maintained essentially constant at an optimum level for the determination of filtration rate. It is obvious that the 2 clear-

TABLE I.

Comparisons of the Thiosulfate and Creatinine Clearances in the Acidotic Dog Over a Range of Plasma Thiosulfate Concentrations.

Exp. No.	Dog No.	Total concurrent time, min.	Arterial plasma concentration				Urine		Rate of excretion		Clearance		
			pH	Bicarbonate, mM/l	Creatinine, mg%	Thiosulfate, mg%	Flow, cc/min.	pH	Titratable acid, mEq./min.	Ammonia, mEq./min.	Creatinine, cc/min.	Thiosulfate, cc/min.	Ratio, Thio./Cr.
1	1	80-90	7.31	13.6	27.8	19.5	2.0	5.05	0.065	0.062	64.0	61.7	0.96
		90-100			26.3	18.3	2.0	5.01	0.074	0.071	74.3	72.8	0.98
		115-125	7.31	13.9	25.5	35.3	10.9	5.00	0.079	0.075	80.4	82.8	1.03
		125-135			25.3	34.7	10.2	5.09	0.070	0.071	79.5	79.8	1.01
		150-160	7.33	13.9	25.6	52.0	11.9	5.34	0.053	0.069	81.3	82.5	1.02
		160-170			26.4	51.1	11.9	5.32	0.054	0.069	80.8	83.4	1.03
2	2	110-120	7.29	11.0	27.4	18.6	5.6	5.10	0.060	0.085	61.3	60.2	0.98
		120-130			26.6	17.8	7.5	5.06	0.063	0.087	63.9	61.6	0.97
		150-160	7.29	11.7	27.4	36.1	11.3	5.17	0.061	0.083	68.8	71.1	1.03
		160-170			28.0	35.9	8.6	5.17	0.060	0.081	67.0	68.7	1.03
		185-195	7.31	11.6	28.9	54.8	8.0	5.24	0.057	0.085	66.1	69.0	1.04
		195-205			29.1	55.6	7.8	5.27	0.053	0.083	65.5	65.8	1.00

[4] Pitts, R. F., and Lotspeich, W. D., *Am. J. Physiol.*, 1946, **147**, 138.

TABLE II.

Experiments Illustrating the Lack of Effect of the Administration of Thiosulfate on Glomerular Filtration Rate and Minimum Effective Renal Plasma Flow in the Acidotic Dog.

Exp. No.	Dog No.	Total concurrent time, min.	Arterial plasma conc.					Urine		Rate of excretion		Clearance			Clearance ratio		
			pH	Bicarbonate, mM/l	Creatinine, mg%	Thiosulfate, mg%	*p*-Amino hippurate, mg%	Flow, cc/min.	pH	Titratable acid, mEq./min.	Ammonia, mEq./min.	Creatinine, cc/min.	Thio. sulfate, cc/min.	*p*-Amino hippurate, cc/min.	Thio. / Cr.	Cr. / P.A.H.	Thio. / P.A.H.
3	1	110-120			29.9		1.05	2.6	5.31	.048	.077	74.4		248		.30	
		120-130	7.20	9.8	29.4		1.12	2.4	5.26	.050	.075	71.1		234		.30	
		130-140			30.2		1.19	2.1	5.19	.050	.081	74.1		238		.31	
		170-180			30.6	51.5	1.38	2.0	4.99	.090	.079	75.4	74.0	238	.98	.32	.31
		180-190	7.22	10.0	30.5	51.3	1.29	2.5	4.98	.098	.079	78.9	77.6	265	.98	.30	.29
		190-200			30.6	52.4	1.22	2.4	4.99	.088	.076	78.9	75.2	268	.95	.30	.28
4	1	115-125			23.7		1.69	2.9	5.72	021	.069	74.2		234		.32	
		125-135	7.33	14.5	23.8		1.69	5.4	6.10	.010	.055	71.6		217		.33	
		135-145			24.0		1.69	6.5	6.26	.008	.048	74.8		221		.34	
		165-175			24.5	28.2	1.75	2.9	5.00	.064	.074	68.3	66.2	197	.97	.35	.34
		175-185	7.36	15.1	25.1	25.9	1.73	1.5	4.92	.070	.076	68.1	66.7	198	.98	.34	.34
		185-195			25.1	24.9	1.68	1.2	4.86	.082	.078	73.2	71.0	217	.97	.34	.33
5	1	100-110				41.9	1.63	3.8	5.37	.006	.092		77.1	228			.34
		110-120	7.27	11.4		42.4	1.72	2.0	5.25	.005	.093		71.3	208			.34
		120-130				41.7	1.70	1.3	4.84	.007	.094		74.0	219			.34
		150-160			36.3	39.7	1.59	8.0	5.08	.087	.085	76.5	72.9	232	.95	.33	.31
		160-170	7.27	11.6	35.9	38.9	1.58	10.9	4.95	.106	.084	78.4	77.8	233	.99	.34	.33
		170-180			36.5	38.7	1.58	5.2	5.08	.088	.080	81.0	78.6	232	.97	.35	.34
6	1	90-100				41.7	1.46	7.5	4.71	.008	.076		71.8	219			.33
		100-110	7.29	14.6		39.8	1.46	3.6	4.84	.008	.074		70.4	215			.33
		110-120				38.7	1.47	1.6	4.69	.008	.074		65.9	195			.34
		120-130			27.9	37.8	1.62	1.9	4.92	.085	.078	73.5	74.1	213	1.01	.35	.35
		130-140	7.29	14.4	28.3	37.6	1.61	4.3	4.88	.088	.078	70.7	71.9	214	1.02	.33	.34
		140-150			28.9	38.4	1.62	4.7	4.87	.094	.076	73.9	73.5	213	1.01	.35	.35

ances are in essential agreement, and that the thiosulfate clearance is independent of plasma level over a 3-fold range of concentration. For these reasons we feel that the thiosulfate clearance gives as accurate an estimate of the quantity of fluid filtered through the glomeruli as does the creatinine clearance. The severity of the acidosis in the animals used in these experiments is indicated both by the low arterial pH values, and by the reduced plasma bicarbonate concentrations. As a consequence of the acidosis the urines were highly acid and ammonia and titratable acid were excreted at accelerated rates. It is significant that the buffering capacity of the urinary creatinine accounts for over 90% of the titratable acid eliminated.

Lack of effect of thiosulfate on glomerular filtration rate and minimum effective renal plasma flow. It is apparent from Experiments 3 and 4 in Table II, that the administration of thiosulfate is without significant effect on filtration rate and minimum effective renal plasma flow. Throughout both experiments these renal variables were measured respectively by the creatinine clearance and by the *p*-aminohippurate clearance. The initial 3 periods of each experiment constitute the control observations. During the final 3 periods thiosulfate was infused at such a rate that its clearance could be used as an accurate measure of filtration rate. No really significant change occurred in either filtration rate or renal plasma flow as a result of the infusion of thiosulfate, slight increases in Experiment 3 being offset by slight decreases in Experiment 4. Identical experiments on 2 other animals yielded similar results.

Advantage of thiosulfate over creatinine in experiments in which titratable acid is to be measured. It was noted in connection with Experiments 1 and 2 that over 90% of the observed urinary titratable acid could be ascribed to the buffering action of creatinine. Experiments 5 and 6 emphasize the fact that the administration of creatinine very markedly increases the rate of elimination of titratable acid in the acidotic animal. In the initial 3 periods of each experiment little buffer substance was present in the urine, and as a consequence the rate of excretion of titratable acid was low, amounting only to 0.005 to 0.008 milliequivalents per minute. Following the administration of creatinine the rate of excretion of titratable acid increased more than 10 times to values ranging from 0.085 to 0.103 milliequivalents per minute. Since the urinary pH did not change, the increased elimination of titratable acid derived solely from the increased elimination of creatinine.

Experiments 3 and 4 in Table II illustrate an interesting phenomenon which we have occasionally observed, namely, that the urine may not be especially acid even though the extent of the reduction of the alkali reserve is great (see especially Experiment 4). The excretion of titratable acid was moderate in this experiment although the urine contained much buffer. Following the administration of thiosulfate, the urine pH fell and the excretion of titratable acid increased proportionally. This effect on urine pH is not peculiar to thiosulfate for we have observed it following the administration of sodium phosphate, sodium *p*-aminohippurate and even sodium chloride in the dog. At the moment we have no adequate explanation for it. It is by no means universally observed.

Summary and Conclusions. The data presented above confirm the observations of Gilman *et al.*[1] that the thiosulfate clearance may be used as a valid measure of glomerular filtration rate in the dog, and extend these observations by showing that it is applicable to animals with disturbed acid base relationships as well as to normal animals. Because the administration of thiosulfate has no significant effect on glomerular filtration rate (creatinine clearance) and minimum effective renal plasma flow (*p*-aminohippurate clearance) the thiosulfate clearance may be used in studies on renal function in acidotic animals with minimal disturbance of these discrete renal variables. Since thiosulfate is not a buffer, the administration of this substance does not greatly alter the rate of excretion of titratable acid. This virtue is likewise possessed by mannitol and inulin, but the simplicity and accuracy of the thiosulfate analysis argues strongly in its favor.

15752

Return Extrasystoles.*

David Scherf.

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In previous reports it has been demonstrated that "return extrasystoles" are observed in dogs during auriculo-ventricular rhythm and simultaneous vagus stimulation or following fatigue of the conduction system by a short lasting tachycardia.[1,2] The resulting disturbance of rhythm closely resembled tracings which were registered from patients in rare cases.[3,4] Whenever the auricles, during auriculo-ventricular rhythm, contracted at a certain time *after* the QRS complexes the return extrasystole was observed.

Of importance is the answer to the problem whether the longitudinal dissociation is always present as an intrinsic quality of the specific conduction system between auricle and ventricle so that the reentry mechanism works whenever the R-P interval reaches a certain length or whether some damage of the conduction system must be added. This would consist in the experiments just mentioned, in vagal stimulation or fatigue. An answer may be possible when more experimental or clinical instances of this arrhythmia become known, particularly when more conditions are studied under which this disturbance of rhythm occurs.

In view of the paucity of experimental observations on this subject 2 more situations, in which the phenomenon of return extrasystoles was observed will be described in this report. It was seen (1) during direct warming of the auriculo-ventricular node of the dog's heart *in situ* by a thermode, and (2) during depression of the sinus and the auriculo-ventricular node by mechanical stimulation of the sinus node area.

(1) *Warming of the auriculo-ventricular node.* These experiments were described in a previous communication in this journal.[5]

The wall of the right auricle was opened after clamping both venae cava; the area of the auriculo-ventricular node near the orifice of the coronary sinus vein was warmed with a thermode. The warming took place within 30 seconds after clamping of the veins was started, that is, at a time when a high degree of anoxia could not have as yet developed.

This experiment was performed in 17 dogs. Return extrasystoles were observed each time in these experiments when an auriculo-ventricular rhythm appeared in which the auricles contracted after the ventricles during the warming. This happened 4 times.

Fig. 1a shows a tracing obtained in such an experiment. Before the warming the vagi were divided in the neck. During the warming a relatively slow auriculo-ventricular rhythm appeared. Deep, peaked inverted P waves are visible after the QRS complexes. Whenever the auricles contracted 0.14 second or later after the ventricles, a return extrasystole, due to a reentry mechanism appeared. The intraventricular conduction of the return extrasystoles was aberrant. Such extrasystoles appear after the second, fifth, sixth and seventh auriculo-ventricular beat in the tracing. Such return extrasystoles were absent after all the other beats unless the R-P interval was long.

Fig. 1b shows the same phenomenon in another experiment of the same type. The warming caused at first a tachycardia and the P waves were not visible. Here again return extrasystoles appeared when the auricles contracted after the ventricles and

* A portion of the costs was defrayed by a grant from the United Hospital Fund.

1 Scherf, D., and Shookhoff, C., *Wien Arch. inn. Med.*, 1926, **12**, 501.

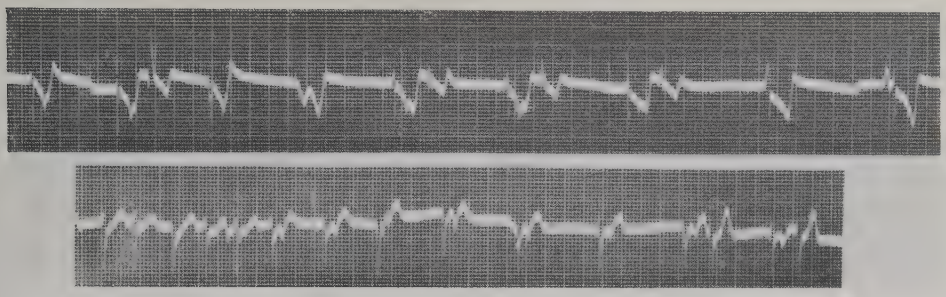
2 Scherf, D., *Arch. int. Med.*, 1941, **67**, 372.

3 Drury, A. N., *Heart*, 1924, **11**, 405.

4 Decherd, G., and Ruskin, A., *Texas Rep. Biol. and Med.*, 1943, **1**, 299.

5 Scherf, D., Proc. Soc. Exp. Biol. and Med. 1944, **56**, 220.

a



b

FIG. 1a.
Return extrasystoles which appeared during warming of the auriculo-ventricular node.
FIG. 1b.
Return extrasystoles during warming of the auriculo-ventricular node, appearing regularly with a long R-P interval.

TABLE I.

Experiment of Feb. 28, Fig. 1b		Experiment of Feb. 28, Fig. 1a		Experiment of April 18	
R-P	P-R	R-P	P-R	R-P	P-R
0	0	12	19	0	0
0	0	14	17	13	10
9	0	11	20	12	0
16	10	11	21	14	9
0	0	11	21	8	0
0	0	10	22	9	15
0	0	9	24	0	0
0	0	10	22	16	9
0	0	9	24	0	0
24	9	7	0	0	0
12	0	7	0	7	0
11	0	7	0	4	0
10	0	8	0	6	0
10	0	8	0	0	0
11	0	10	24		
10	0	7	0		
12	16	7	0		
12	17				
10	0	13	0		
0	0	14	20		
0	0	12	0		
		4	0		
		14	20		
		12	25		
		14	22		
		0	0		
		0	0		

the R-P interval reached a length of 0.12 second. Premature contractions did not follow any other beat. Therefore a reentry mechanism can be assumed.

The tracings from the 2 other experiments of this series showed the same picture.

Table I gives the measurements obtained in 3 tracings with return extrasystoles. They show clearly that a return extrasystole occurs when the R-P distance reaches a certain length. The reciprocal duration of the R-P distance and of the following R-P interval is evident. All beats in which the P waves were hidden in the QRS complex or in which positive P waves preceded the QRS complex are designated with "O". Following beats of this type return extrasystoles were absent, for obvious reasons.

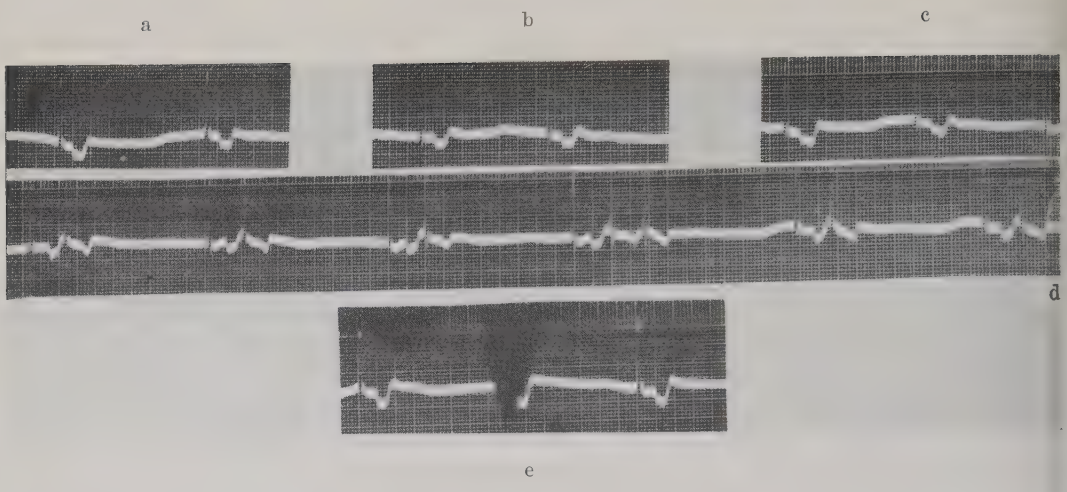


FIG. 2.
Return extrasystoles during reflex inhibition of the sinus- and the auriculo-ventricular node.

(2) *Depression of sinus and auriculo-ventricular node.* This phenomenon was described in a previous paper in this journal.[6] The subepicardial injection of hypertonic solutions of sodium chloride or calcium chloride, as well as digitalis or strophanthin in the sinus node area of the right auricle led in a certain percentage of experiments on dogs to a depression of the sinus node. Frequently the deeper auriculo-ventricular nodal centers were also inhibited. This depression was particularly pronounced in one experiment in which during the auriculo-ventricular rhythm the auricles contracted *after* the ventricles.

The heart of the dog was exposed during nembutal-morphine anesthesia. Both vagi were severed in the neck. Then 0.1 cc of a 5% solution of calcium chloride was injected subepicardially in the area of the head of the sinus node. Immediately a marked bradycardia appeared and an auriculo-ventricular rhythm was registered with an R-P interval of 0.16 second (Fig. 2a). Without any other means the length of the R-P distance gradually increased in the following minutes. In Fig. 2b it is 0.17 second and in Fig. 2c it measures 0.18 second. As soon as it reached the value of 0.20 second, bigeminy and trigeminy appeared (Fig. 2d). About 2 minutes later and 3.5 minutes after the injection, the extrasystoles disappeared and the R-P interval at that time was again shortened to 0.18 second (Fig. 2e). The occurrence of trigeminy due to return extrasystoles was described before.[2]

Discussion. These experiments show 2 new ways which lead to the appearance of return extrasystoles. In both, however, a change of the condition of the auriculo-ventricular conduction system cannot be completely excluded. This damage could be due to anoxia in the first experiments in which the exposed auriculo-ventricular node was warmed although it is rather improbable in view of the early appearance of the return extrasystoles and the shortness of the P-R and R-P intervals. In the second type of experiment the depression of the nodal centers may also have extended to the deeper sections of the specific tissue.

Conclusion. Two new methods are described which lead to the appearance of return extrasystoles during auriculo-ventricular rhythm in the dog. Such extrasystoles appeared during direct warming of the exposed auriculo-ventricular node *in situ* and during depression of the nodal centers by the subepicardial injection of a hypertonic solution of calcium chloride.

[6] Scherf, D., PROC. SOC. EXP. BIOL. AND. MED., 1946, **61**, 286.

15753

Stimulation of Sporogenic and Nonsporogenic Bacteria by Traces of Penicillin or Streptomycin

HAROLD R. CURRAN AND FRED R. EVANS. (Introduced by G. E. Holm.)

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The remarkable therapeutic efficacy of penicillin and streptomycin is dependent upon their low toxicity to tissues and their unusual capacity to inhibit or kill, *in vivo*, a wide variety of pathogenic organisms.

That the biological activity of these antibacterial agents is not limited to their bactericidal or bacteriostatic properties may be readily demonstrated, although this fact seems to have been overlooked by most microbiologists and clinicians. The bacteria-controlling property of these drugs has for the most part obscured the phenomenon of growth stimulation associated with low concentrations of the drugs. Evidence of a so-called biphasic action of penicillin has been reported by Miller, Green, and Kitchen[1] who noted that certain concentrations of penicillin in broth containing *Staphylococcus aureus* induced upon incubation, increased turbidity over the control containing no penicillin. The effect was irregular at 37°C but quite consistent at 24°C.

It is the purpose of this paper to show that suitably diluted solutions of either penicillin or streptomycin, when incorporated in a nutrient medium, exert a definite stimulating effect upon the growth of many bacterial species. Stimulation of this nature was first observed in a spore-forming species with tomatin as the test substance; our observations were then extended to penicillin and streptomycin and widened to include a rather large group of spore-forming species and 2 nonspore-forming pathogenic species.

When spore-forming species were used, washed spores were seeded uniformly into tubes of sterile (autoclaved) skim milk to yield a final concentration of approximately 50,000 viable spores per milliliter. The inoculated samples were heated at 95°C for 15 minutes and cooled, after which distilled water dilutions of the antibiotics*,† were thoroughly mixed with the inoculated samples. These samples together with the controls, which received no antibiotics but were manipulated similarly, were then incubated, usually at 30°C and observed at frequent interva's for the first visible indication of growth. In the nonsporing species a small loopful of a 17-hour tomato broth culture served as inoculum for each tube of medium. The requisite dilutions of reagents were made up from the powder just prior to use. Two or more controls were included in each series to take account of chance variations. The data were essentially reproducible although in some samples unexplained discrepancies occurred.

Examination of the data (Table I) shows that both penicillin and streptomycin were for most strains stimulatory at one or more concentration levels. The degree of stimulation varied from a few hours to several days as measured by first visible evidence of growth‡ between the test and control samples. Film preparations made at one or more stages in the development of the cultures, in most instances revealed a positive correlation between visible indication of stimulation and cell numbers. Greatest stimulation was found in *Bacillus megatherium*, while in *Bacillus mycoides* and certain strains of *Bacillus cereus* it was not apparent. In *Clostridium perfringens* (2 cultures) stimulation was slight and frequently irregular. The

[1] Miller, W. S., Green, C. A., and Kitchen, H., *Nature*, 1945, **155**, 210.

* Penicillin sodium obtained from Chas. Pfizer & Co., Brooklyn, N.Y.

† Streptomycin hydrochloride obtained from Merck & Co., Rahway, N.J.

‡ Perceptible thickening, surface ring of peptonized milk or gas bubble.

TABLE I. Effect of Traces of Penicillin or Streptomycin upon the Growth of Bacteria in Milk.

Organism	No.	Incubation temp., °C	Penicillin u/ml				Streptomycin u/ml			
			0.01	0.001	0.0001	0.00001	0.05	0.005	0.0005	
B. cereus	369	30	—	+ 2 hr	+ 2 hr		+	+	+ 8 hr	
" "	401	30	0	+ +	+ 3 hr	+	0	+ + 3 hr	0	
" "	232	30	+ sl	+ sl	0	0	0	+ + 2 hr	0	
B. mycoides	6462	30	0	0	0	0	—	0	—	
B. megatherium	696	30	+	+ + 3 days	—		+ + 5 days	+ +		
B. subtilis	L.B.	30	—	+ + 4 hr	+	0	+ + 6 hr	+ +	+	
" "	6051	30	—	—	0		+ + 6 hr	0	0	
" " *aterrimus*	230	30	0	0	0	+ 2 hr	+ + 4 hr	+	0	
B. stearothermophilus	1518	52	—	—	0					
" "	07	52	—		+ +	+ 7 hr	+		+ +	4 hr
Cl. perfringens	846	37	—	0	+ +	+ sl	—	+ + sl	+ +	3 hr
" "	3626	37	—	0	0	0	+ + sl	0		
	3679*	30	—	—	+ 1 2 days	+		+ + 4 hr	0	
Strep. agalactiae		37	+ sl	+ sl	0	0	+ + sl	+ + sl	+ sl	
Staph. aureus		37	—	0	+ sl	0	+ + sl	+ + sl	0	

* A proteolytic anaerobe.
0 = first visible evidence of growth approx. equal in time to, — = greater than (retardation), and + = less than control (stimulation).
For the level of concentration which showed maximum stimulation, the time has been indicated. sl = less than 2 hours.

2 nonspore-formers,§ *Streptococcus agalactiae* and *Staphylococcus aureus,* were chosen because of their causal relationship to bovine mastitis. The latter evidenced rather slight and at times irregular stimulation at the 0.001 and 0.0001 levels (u/ml) of penicillin and in most of the streptomycin concentrations. *Strep. agalactiae* was appreciably stimulated through a wider range of penicillin and streptomycin. In general, streptomycin was more frequently stimulatory than was penicillin. It is perhaps significant that the stimulatory effects of penicillin and streptomycin were generally more apparent in the cultures incubated at 30°C than at higher temperatures, which accords with the previously mentioned observation of Miller *et al.*[1] upon the staphylococcus.

In view of the limited number of drug levels used, it should not be supposed that the most favorable concentrations were attained in these experiments or that cultures negative in reaction would not respond at other drug levels. The recorded data were obtained in a skim milk medium; when this medium was enriched by the addition of glucose and yeast extract the stimulating properties of the antibiotics were usually greatly reduced or entirely eliminated.

Welch, Price, and Randall[2] recently reported that streptomycin at certain concentration levels when injected intraperitoneally in mice infected with *Eberthella typhosa* increased rather than decreased the fatality rate. This suggested to the authors the possibility of stimulation of the infective agent by streptomycin; further strengthening this view was their observation that certain levels of streptomycin in broth, though inhibiting any visible evidence of growth, induced, nevertheless, a greater multiplication of cells than did samples containing lower concentrations of the drug. This report, together with that of Miller *et al.*,[1] came to our attention after most of our observations were completed. The former observations together

§ Recently isolated from acute bovine mastitis by L. A. Burkey, this Bureau.

2 Welch, H., Price, C. W., and Randall, W. A., *J. Am. Pharm. Assn.,* 1946, **35**, 155.

with those here reported indicate that the stimulating activity of penicillin and streptomycin evident *in vitro* is probably operative *in vivo*.

Discussion. By evincing, in suitable concentrations, bacteriostatic or bactericidal activity and at certain much lower concentration levels a stimulatory effect, penicillin and streptomycin conform to a familiar behavior pattern of drugs and chemical reagents. The biphasic action of sulphonamides has been reported.[1] Recognition of the potential stimulatory activity of these drugs further increases the problems of initial dosage and maintenance in the body fluids of proper drug levels. The importance of achieving complete destruction of the infective agent by the initial dosages receives added emphasis. It is apparent also that concentrations of these drugs efficacious as bactericidal or bacteriostatic agents for one species of microorganism, for another, perhaps coexistent infective agent, may be stimulatory. Thus, Eagle *et al.*[3] found that 0.01-0.02 u/ml of penicillin was actively spirochaeticidal, a zone not infrequently stimulating to the spore-forming species employed in our study. Rammelkamp and Keefer[4] reported maximum antibacterial action for *Streptococcus haemolyticus* when the concentration of penicillin in blood and serum was 0.019 u/ml. Evidence[5,6] seems to indicate that part of the therapeutic activity of penicillin is associated with accompanying impurities, a fact which has necessitated in recent years the administration of larger doses to offset the greater refinement of the product; conversely it is not unlikely that the stimulatory concentration level of penicillin and streptomycin would be lowered by increased purification.

Summary. Suitable, low concentrations of penicillin or streptomycin in sterile milk (autoclaved) stimulated the growth of many spore-formers and *Staphylococcus aureus* and *Streptococcus agalactiae*. The degree of stimulation differed with the organism and with some cultures was not apparent in the range of concentrations used; some of the practical implications of growth stimulation by penicillin and streptomycin are briefly discussed.

3 Eagle, H., Magnuson, H. J., and Fleischman, R., *Johns Hop. Hosp. Bull.*, 1946, **79**, 168.

4 Rammelkamp, C. H., and Keefer, C. S., *J. Clin. Invest.*, 1943, **22**, 649.

5 Smith, W. J., *Science*, 1946, **104**, 411.

6 Comm. Med. Res., U. S. P. H. and F. D. A., *J. Med. Assn.*, 1946, **131**, 271.

15754

Studies on Auricular Tachycardia Caused by Aconitine Administration.

DAVID SCHERF.

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Aconitine in minute amounts provokes abnormal stimulus formation in the heart. It has been demonstrated that with particular precautions a long-lasting, regular, bigeminal rhythm can be obtained after the intravenous administration of aconitine in the experimental animal.[1] Studies on the effect of topical application of aconitine on the dog's heart are reported in this paper.

Method. The experiments were performed on 23 dogs. Morphine-nembutal anesthesia was used. The heart was exposed in the usual way. The vagi were severed in the neck. If necessary, the peripheral end was stimulated with the aid of a Cambridge inductorium.

The electrocardiograms were registered in lead II.

* Aided by a grant from the United Hospitals Fund.

1 Scherf, D., *Z. f. d. ges exp. Med.*, 1929, **65**, 198, 222.

Solutions of 0.05% of "potent" aconitine crystals "Merck" were used. The injections were performed with greatest care with the aid of a tuberculin syringe. Even the small amounts of aconitine, which were used in these experiments, if introduced into the auricular cavity, caused a ventricular tachycardia and ventricular fibrillation. One must be careful not to leave any aconitine solution on that part of the auricular surface which is in contact with the ventricular wall because this also leads to a ventricular tachycardia and eventually to ventricular fibrillation. The potency of the solution quickly deteriorates, even when kept in the refrigerator.

Most of the injections were performed on the appendix of the left auricle, a few millimeters below its tip. Injections into the wall of the appendix of the right auricle had the same result.

Experimental results. Injection of 0.05 cc of a solution of 0.05% of aconitine into the wall of the auricles of the dog's heart leads to a prolonged regular auricular tachycardia. The tachycardia begun within 90 seconds after the injection. The rate varied between 196 and 310 per minute. The tachycardia lasted between 40 and 60 minutes, and was therefore sufficiently long to permit certain studies to be made.

In these experiments the following facts could be demonstrated:

1. Faradic stimulation of the right or left vagus nerve during the auricular tachycardia led to a remarkable acceleration of the rate of the auricle in all experiments. This acceleration often amounted to an increase of more than 100%. The acceleration was immediate, and after the end of stimulation, the former rate returned within a few seconds in the form of a rapid but not abrupt decline. The auricular waves remained unchanged in the electrocardiogram, or showed only the slight alterations which would be expected with an increase in rate. No difference could be detected in the results when right and left vagus were stimulated respectively. Observation of the right auricle during the vagus stimulation revealed an immediate disappearance of the strong fluttering movements, and on close observation, weaker, but rapid and regular undulatory movements could be detected. A varying degree of auriculo-ventricular block appeared. Auricular fibrillation, or rapid reexcitation of the auricles with rates up to 3500 per minute was never observed. In only one experiment did auricular fibrillation appear following the injection of aconitine and before stimulation of the vagi.

2. Clamping off the appendix of the auricle into which the injection was made, invariably led to an immediate disappearance of the tachycardia and the reappearance of sinus rhythm. In some instances coronary sinus rhythm with deep inverted P waves was noted; this was soon followed by regular sinus rhythm. Removal of the clamp permitted the tachycardia to reappear within a few seconds.

3. Stimulation of the vagus while the clamp was in position led to a complete cardiac standstill. Only in the clamped-off appendix of the left auricle were fine undulatory movements visible.

4. Cooling the focus of origin of the tachycardia, that is the area where the injection had been made, with a thermode also caused the tachycardia to vanish for the duration of the cooling. The tachycardia recurred immediately and invariably with the removal of the thermode.

Fig. 1a shows at the beginning a regular tachycardia caused by the injection of aconitine in the manner described in the appendix of the left auricle. A rate of 230 auricular and ventricular beats per minute is present. The R waves are so thin that they are scarcely visible. The P waves are tall. After the 10th beat the left vagus was stimulated in the neck with a strong faradic current. The auricular rate immediately increased to 428 beats per minute without any change in the form of the auricular waves. An auriculo-ventricular block appeared and made it easier to observe the auricular electrocardiogram. The vagal stimulation was stopped a few seconds later (first third of the tracing in Fig. 1b, which is a direct con-

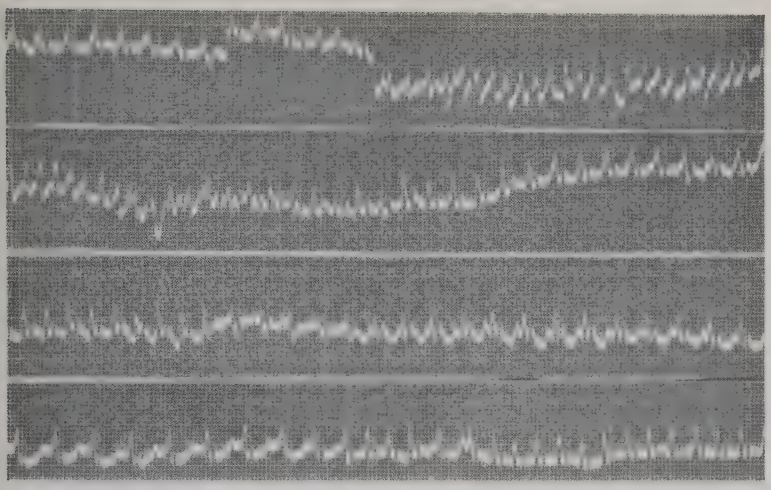
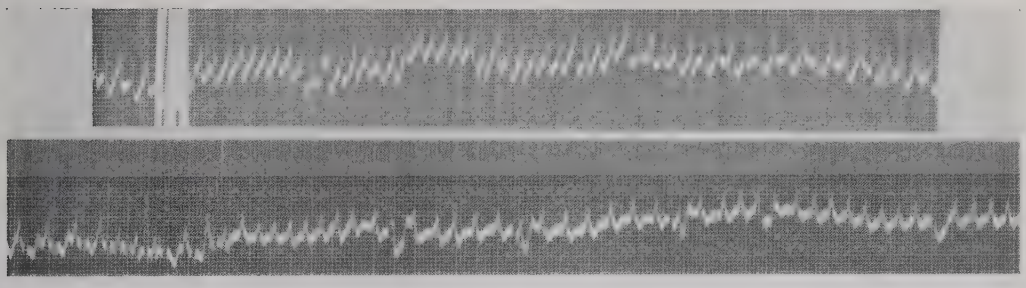


FIG. 1, a-d.
The top tracing (Fig. 1a) shows at the beginning an auricular tachycardia and the effect of vagus stimulation which begins in the middle of the tracing. Fig. 1b shows the end of the vagus stimulation (middle of tracing). In the beginning of tracing 1c the site of stimulus formation was clamped off; in the middle of Fig. 1d the clamp was removed (Lead II).

a



b

FIG. 2a and 2b.
The signal indicates the beginning of vagus stimulation. Fig. 2b shows a slight irregularity of the auricle during vagus stimulation (Lead II).

tinuation of Fig. 1a). Without delay the same tachycardia with a rate of 230 was again registered as in the beginning of Fig. 1a. In the first third of Fig. 1c, the appendix of the left auricle was clamped, so that the site of injection was isolated from the rest of the heart. A sinus rhythm appeared immediately with a rate of 187 beats per minute and soon the rate fell to 156. The clamp was removed a few seconds later (middle of Fig. 1d, which is a direct continuation of Fig. 1c), and an auricular tachycardia reappeared with a rate of 250 beats per minute.

Fig. 2a shows at the beginning a few complexes of a tachycardia with a rate of 200 which appeared following an injection of the same quantity of aconitine into the medial wall of the left auricular appendix. The broad, wide, irregular line represents a signal indicating the beginning of stimulation of the left vagus nerve. During the stimulation of the vagus the auricular rate rose immediately to 461 and there was almost complete inhibition of the auriculo-ventricular conduction. The cessation of vagus stimulation was followed by an immediate recurrence of the

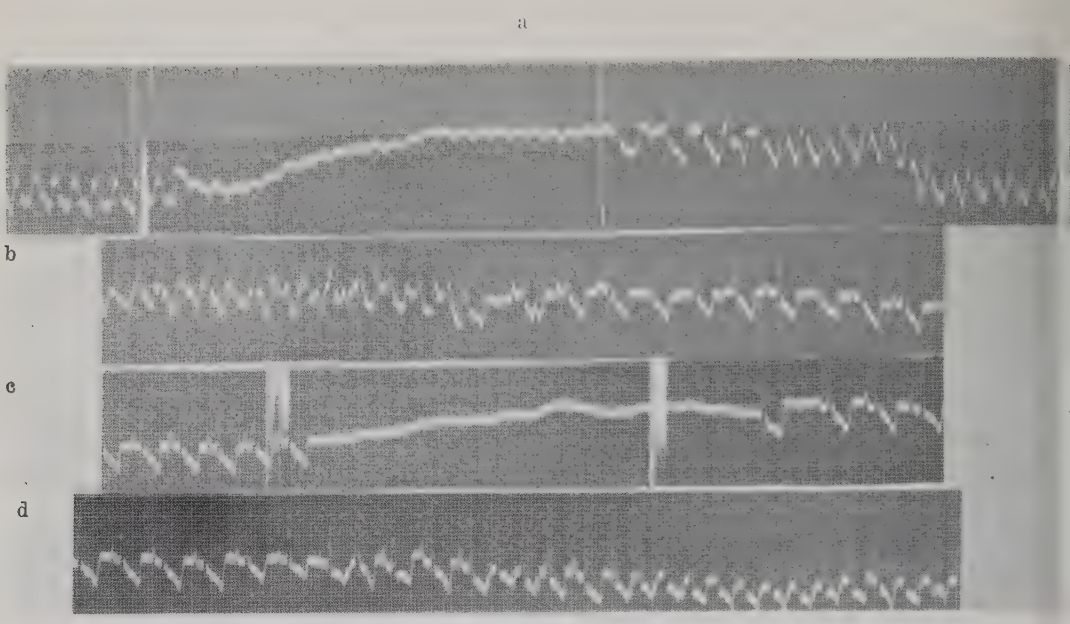


FIG. 3, a-d.
Vagus stimulation during auricular tachycardia (Fig. 3a), clamping off the focus in the right auricle (Fig. 3b), vagus stimulation during presence of clamp (Fig. 3c), and removal of clamp (Fig. 3d) (Lead II).

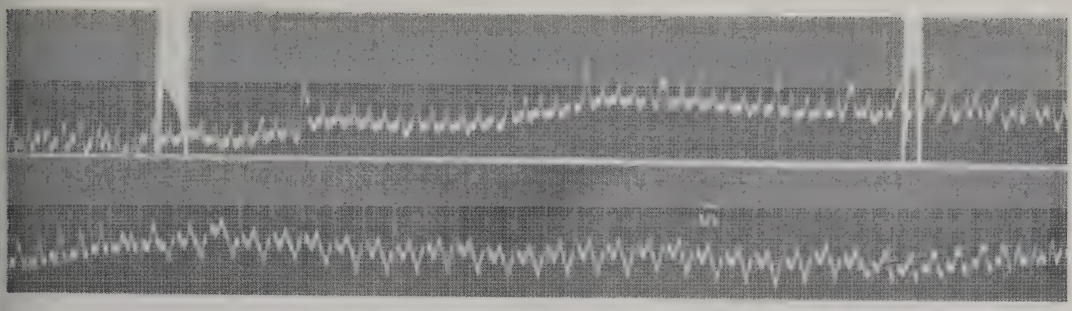
former tachycardia with a rate of 200 beats per minute.

Only rarely was the auricular tachycardia during vagus stimulation irregular. In Fig. 2b, which was secured in another experiment than Fig. 2a, at the beginning of the tracing the auricular rate after administration of aconitine was again 200. During stimulation of the right vagus nerve the rate increased to an average of 428 beats per minute and slight arrhythmias were visible. Temporary interruption of the contact of the stimulating electrode with the vagus nerve could not be excluded.

Fig. 3 is from an experiment in which the aconitine was injected on the tip of the right auricular appendix. A tachycardia with a rate of 272 appeared within a few seconds (Fig. 3a). Faradic stimulation of the right vagus led to an increase of rate to 375 beats per minute (Fig. 3a), and to a complete inhibition of the auriculo-ventricular conduction. The 2 perpendicular white lines represent signals showing the beginning and the end of the vagal stimulation. After stimulation was stopped a short period of 2:1 block is noted (Fig. 3a), and the regular tachycardia with a rate of 280 recurred. Clamping off the right auricular appendix caused a sinus rhythm to appear immediately (Fig. 3b) with a rate of 150. In Fig. 3c, the effect of the vagus stimulation during the clamping, and the existence of a sinus rhythm is demonstrated; a complete standstill of the heart resulted, without any sign of activity in the electrocardiogram. Removal of the clamp permitted the tachycardia to recur immediately in the same form as before with a rate of 214 (Fig. 3d).

Fig. 4 was obtained in an experiment in which the injection was made on the appendix of the left auricle. A tachycardia with a rate of 310 appeared, and the rate increased during the vagus stimulation (see signals) only to 375 (Fig. 4a). Cooling of the site of origin of the tachycardia (the area of injection) led to the immediate disappearance of the tachycardia (Fig. 4b). A regular coronary sinus rhythm with a rate of 187 was present. Interruption of cooling was immediately followed by reappearance of the tachycardia, at the end of Fig. 4b. Usually

a



b

FIG. 4a and 4b.
Vagus stimulation during auricular tachycardia (Fig. 4a) and cooling of area of injection (Fig. 4b) (Lead II).

cooling led to the appearance of sinus rhythm. The response to cooling was, in general, the same as to clamping off of the area of injection.

In most experiments all these measures, such as vagus stimulation, clamping, and cooling, were performed 3 or 4 times in succession, and always with the same results.

Discussion. Of paramount importance in the evaluation of these experiments is the question as to whether the tachycardias caused by the injection of aconitine represent an auricular tachycardia of the "essential paroxysmal tachycardia" type, or whether we are dealing with auricular flutter. This differentiation is often impossible and in recent years many arguments have been presented in favor of the identity of these conditions. It is the opinion of the author that for the time being a separation is necessary, and that the facts in favor of their identity are too few and not really crucial.

The tracings appear similar to those published by many authors as examples of experimental auricular flutter.[2-5] The isoelectric line between the single auricular waves is short or absent. Flutter rates in the dog's auricles however, were found to vary between 345 and 580[6] while the rate in the experiments reported in this paper was definitely below this range before vagus stimulation and within it during stimulation. Against the diagnosis of a simple tachycardia like the essential paroxysmal tachycardia in man there is the observation that stimulation of either vagus nerve with even the strongest faradic current never caused sudden ending of the tachycardia. This sudden abolition of an auricular tachycardia is seen however in only a certain percentage of essential paroxysmal tachycardia, and absence of this effect does not invalidate the diagnosis. It is entirely possible that the inability to end the tachycardia by vagal stimulation is due to the special conditions which led to the tachycardia in this experiment. Against the existence of auricular flutter in these tracings, the argument may be used that it was never possible, even with the strongest current, to change the auricular tachycardia into auricular fibrillation by vagus stimulation. One easily succeeds in doing this in auricular flutter caused by other means.[4,7] The phenomenon of rapid reexcitation with auricular rates between 1500 and 3500 per minute rarely occurs during vagus stimulation

2 Lewis, T., Drury, A. N., and Bulger, H. A., *Heart*, 1921, **8**, 83.

3 Lewis, T., Drury, A. N., and Bulger, H. A., *Heart*, 1921, **8**, 141.

4 Rothberger, C. J., and Winterberg, H., *Arch. f. d. ges. Physiol.*, 1914, **160**, 42.

5 Scherf, D., *Z. f. d. ges. exp. Med.*, 1928, **61**, 30.

6 Lewis, T., Feil, H. S., and Stroud, W. J., *Heart*, 1918/20, **7**, 191.

7 Lewis, T., Drury, A. N., and Iliescu, C. C., *Heart*, 1921, **8**, 311.

unless the flutter rate before the stimulation is over 400 per minute.[7] The gradual but rapid increase and decline of the auricular rate which has been observed in instances of auricular flutter during and after vagus stimulation[4] is very similar to the phenomenon reported here.

In view of all these facts, it seems that a definite decision is impossible and the presence of either type of auricular tachycardia cannot be definitely ruled out.

A paradoxical increase in the formation of auricular and ventricular extrasystoles after vagus stimulation in the dog following intravenous injection of aconitine has been described as a regular phenomenon. Intravenous injection of choline preparations had the same effect.[1] The increase in the number of auricular extrasystoles in these experiments occurred however only *after* the stimulation of the vagus; during the stimulation only the usual inhibition of the auricles with standstill was seen.

The phenomenon of reexcitation and the marked increase of rate of the auricles, during vagus stimulation was used by Lewis and his associates in brilliant studies[3,6,7] as one of the more important facts supporting the theory of circus movement. The shortening of the refractory phase during the stimulation of the vagus, which may amount to more than 1/5 of the normal, abolishes barriers in the form of islands of refractory muscle in the way of the circulation "Mother" wave and causes this wave to use a less sinuous path and therefore to circulate faster. Another possibility considered by Lewis would be the utilization of another path which is shorter than the original one around the orifices of the great veins.

One must concede that the circus movement theory explains both phenomena, the moderate increase of rate during vagus stimulation, as in the experiments reported here, and the appearance of the rates of over 3000 per minute, better than any other theory.[8] A simple increase of the rate of stimulus formation is difficult to accept in view of the high rates which occur in rapid reexcitation and in view of the known inhibitory action of the vagus on the heart. Rothberger and Winterberg, who explained flutter by a rapid stimulus formation in a single center, also tried to correlate the phenomenon of rapid reexcitation during vagus stimulation with a shortening of the refractory period; the finer mechanisms however are still unexplained. There is no information about the influence of vagus stimulation on the formation of stimuli in the centers, and it is possible that an increase of the rate of stimulus formation during vagus stimulation is a typical consequence of the shortening of the refractory period.

The observation that clamping off the site of origin of the tachycardia or cooling the point of injection of the aconitine abolished the tachycardia and immediately reintroduced sinus rhythm is of interest in connection with the 2 viewpoints regarding the mechanism of origin of auricular flutter. Still more important is the fact that interruption of the cooling causes the tachycardia immediately to reappear. These observations are not easily explained by a circus mechanism. It has been maintained that localized circus movements occur and cause localized auricular fibrillation which can be interrupted by clamping off or cooling a small area.[9] The path of such a small circulating wave was estimated to use a circuit with a diameter of only 2.5 to 3.8 mm.[7] If such a localized circus movement exists in these experiments however, it is hard to understand how interruption of the cooling is immediately followed by the reappearance of the same tachycardia as before. Without the assumption of a heterotopic focus of stimulus formation, this observation is difficult to explain.

Thus, in conclusion, one may state that if we are dealing with auricular flutter in these experiments, the effect of the cooling of the area into which the solution of aconitine was injected argues against the assumption of a circus movement. If we are dealing with a heterotopic tachycardia, the appearance of an acceleration of rate during

[8] Garrey, W. E., *Physiol. Rev.*, 1924, **4**, 215.

[9] McWilliam, J. A., *Proc. Roy. Soc., London, Ser. B*, 1918, **90**, 302.

vagus stimulation not based on a circus movement mechanism is of interest.

Summary. Focal application of aconitine to the dog's auricle in the form of a subepicardial injection is followed by the appearance of a prolonged regular tachycardia with a rate of approximately 200 to 300 beats per minute. Faradic stimulation of the vagus nerves in the neck always leads to a remarkable increase of rate of the auricles. Auricular fibrillation or the phenomenon of rapid reexcitation were never observed during the vagus stimulation.

Separation of the site of injection from the rest of the heart by clamping abolishes the tachycardia; it regularly reappears on removal of the clamp. Cooling of the site of injection by a thermode also stops the tachycardia, and it reappears immediately when cooling is discontinued.

The tachycardia shows characteristics of auricular flutter, but auricular tachycardia of the type "essential paroxysmal tachycardia" cannot be ruled out.

The results of the experiments cannot be explained under the assumption that the tachycardia is caused by a circus movement. Therefore the increase of rate during the vagus stimulation requires another explanation than the one given by Lewis.

15755

A Method for Intrathoracic Operation on the Rat.*

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Positive control of intrapulmonary tension and oxygenation is highly desirable for intrathoracic operations on any animal. The technic for larger animals, such as dogs, is well standardized, but because of the small size of the laboratory rat, none of the methods could be satisfactorily used in intrathoracic operations on that animal.

In connection with problems involving pneumonotomy and pneumonectomy in the rat, a simple and satisfactory method of positive pressure administration of oxygen has been evolved utilizing tracheal intubation. The rat is anesthetized with pentobarbital sodium intraperitoneally at a dosage of 30 mg per kg of body weight. In combination with this is given scopolamine hydrobromide, in a dosage of 0.6 mg for a 300 to 400 g rat.[1] The latter drug was found to increase rapidity and depth of anesthesia, reduce tracheal secretions and counteract the respiratory depression that pentobarbital sodium tends to produce. The pharynx is cleared by suction and the trachea is intubated with a special tube to be described later. The end of the tube is then connected to the oxygen line and valve apparatus. Respiration is not artificial but is performed in the natural manner with the rat working against a variable pressure water valve.

The intratracheal tube is constructed of plastic tubing† having an outside diameter of 0.08 inch (0.20 cm) and an inside diameter of 0.045 inch (0.11 cm) (Fig. 1). One end is sealed off by rotating in a gentle flame and molding with the fingers, and a slight point is produced by grinding with an emery wheel. Just above the point 3 small holes, staggered around the circumference, are cut with fine scissors and ground smooth with

* Work done in the laboratory of, and under the direction of, Dr. George M. Higgins, Division of Experimental Medicine, Mayo Foundation, Rochester, Minn.

[1] Holck, H. G. O., Dosage of Drugs for Rats, in Griffith, J. Q., Jr., and Farris, E. J., *The Rat in Laboratory Investigation*, Philadelphia, J. B. Lippincott Company, 1942, chap. 13, pp. 297-350.

† Transflex, courtesy of the manufacturer, The Irvington Varnish and Insulator Company, Irvington, N.J.

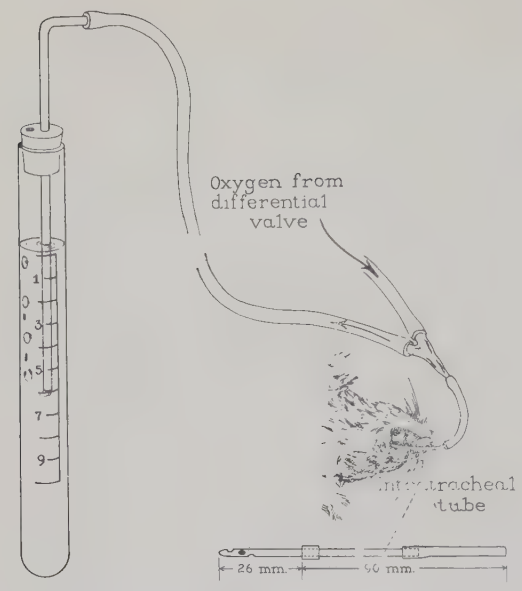

FIG. 1.
Positive pressure water valve, intracheal tube and method of connection. The test tube is conveniently supported by a buret clamp attached to a ring stand. See text.

a small dental burr. A cuff of rubber tubing is placed with its lower edge 26 mm above the tip, in order to prevent insertion of the tube beyond the carina. A similar piece of tubing is slipped over the upper end of the plastic tube to serve as a connector and both are cemented in place with rubber cement. A stiff wire stylet is used during insertion.

Intubation must be done under direct vision in the rat owing to the extreme mobility of the laryngeal structures. A satisfactory "laryngoscope" can be a bivalve electric otoscope with half of the objective removed or an ordinary electric otoscope with the objective cut in half lengthwise (Fig. 2). The anesthetized rat is placed on its back on a rat board, the tongue is pulled well out, the upper jaw is held down by a loop over the front teeth and the tube is inserted between the vocal cords with the aid of the converted otoscope.

The positive pressure apparatus consists of a simple water valve (Fig. 1). A large test tube about 20 cm long is fitted with a 2-holed rubber stopper and filled about two-thirds full of water. A glass tube about 6 mm in outside diameter slides through one of the holes in the stopper; the depth of immersion of the tube determines the amount of positive pressure. The second hole in the stopper allows escape of the excess gases. A centimeter scale marked on the side of the test tube allows exact settings. A glass Y tube completes the apparatus. One arm is connected to the differential valve on the oxygen tank, the second arm is connected to the water valve and the base of the Y is connected to the rubber tubing on the end of the intratracheal tube. It is very important to keep the last connection short in order to reduce the amount of physiologic dead space created by the intratracheal tube.

In using the apparatus a positive pressure of 1 to 2 cm of water is used until the thorax is about to be opened. For pneumonectomy or any operation in which maintaining a fully expanded lung is not important, the pressure is increased to 5 or 6 cm of water during the period when the thorax is open. If continued full expansion of the lung is desired, a pressure of 7 to 8 cm of water is necessary but this sometimes causes leakage of air into the mediastinum or the opposite pleural cavity, so that the lower pressures are generally more practical. Inflation of

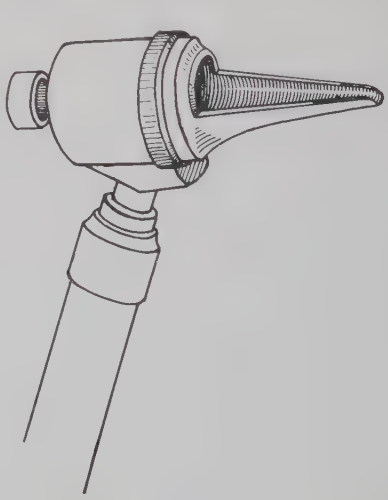

FIG. 2.
Converted electric otoscope used for the insertion of the intratracheal tube. Most of the top half of the objective has been cut away to form a miniature "laryngoscope."

partially collapsed lung may be obtained by briefly pinching off the rubber tubing leading to the water valve.

Oxygen flow is carefully regulated by means of the differential valve on the oxygen tank. When the flow is properly regulated, oxygen bubbles gently through the water valve at all times except during inspiration, when the flow is temporarily interrupted. Even during inspiration there should not be much rise of water level in the glass tubing of the valve.

For satisfactory operation, obstruction of the intratracheal tube must be avoided. If there is excessive trauma during intubation, blood is almost sure to clog the tube. Rats that have colds or mild chronic pneumonitis should not be used because excess secretion clogs the intratracheal tube. Suction or repeated insertion of the stylet will sometimes free a clogged intratracheal tube.

We have used the method described in this paper in more than 300 thoracotomies on rats, about 200 of which were pneumonectomies. It has proved satisfactory and easy to use once the technic has been mastered. Best results were obtained when temperature and humidity were moderate. Hot, humid days seemed to cause a definite increase of the operative mortality rate.

Summary. For performance of intrathoracic operations on the rat pentobarbital sodium and scopolamine were found to be effective anesthetic agents. A plastic intratracheal tube was devised for use in such operations and has proved to be satisfactory. A simple water valve apparatus for administration of oxygen under positive pressure was devised for use with the intratracheal tube.

15756

Intravascular and Intracardiac Pressure Recording in Man: Electrical Apparatus Compared with the Hamilton Manometer.*

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An electrical apparatus has been used for recording blood pressures, (peripheral artery, right auricle, right ventricle and pulmonary artery), pleural pressure, and mask pressure in man. The method was tested for accuracy and fidelity in reproduction of the pressure tracings by comparison with the Hamilton manometer[1] used extensively for the recording of right heart pressure.[2,3] Records were obtained of simultaneous tracings on both systems, permitting a beat to beat study (Fig. 1, A and B). A record was also taken on the Hamilton manometer with the electrical recording off (Fig. 1, C).

The electrical pressure pick-up unit used was the Clark Pressure Capsule.† This capsule consists of 2 small coils mounted in a small round metal case (38 mm in diameter and 12.5 mm thick) and separated by a

* Under contract with Aero-Medical Laboratory, Wright Field, Dayton, Ohio. Additional support was provided by the Commonwealth Fund and the Life Insurance Medical Research Fund Gift for Study of Action of Certain Cardiovascular Drugs.

1 Hamilton, W. F., Brewer, G., and Brotman, I., *Am. J. Physiol.*, 1934, **107**, 427.

2 Cournand, A., Lauson, H. D., Bloomfield, R. A., Breed, E. S., and Baldwin, E. de F., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 34.

3 Bloomfield, R. A., Lauson, H. B., Cournand, A., Breed, E. S., and Richards, D. W., Jr., *J. Clin. Invest.*, 1946, **25**, 639

† Clark Capsule, designed by James C. Clark, Wright Field, Dayton, Ohio. Aerotronics Inc., Camden, Ohio, licensed to manufacture the capsule.

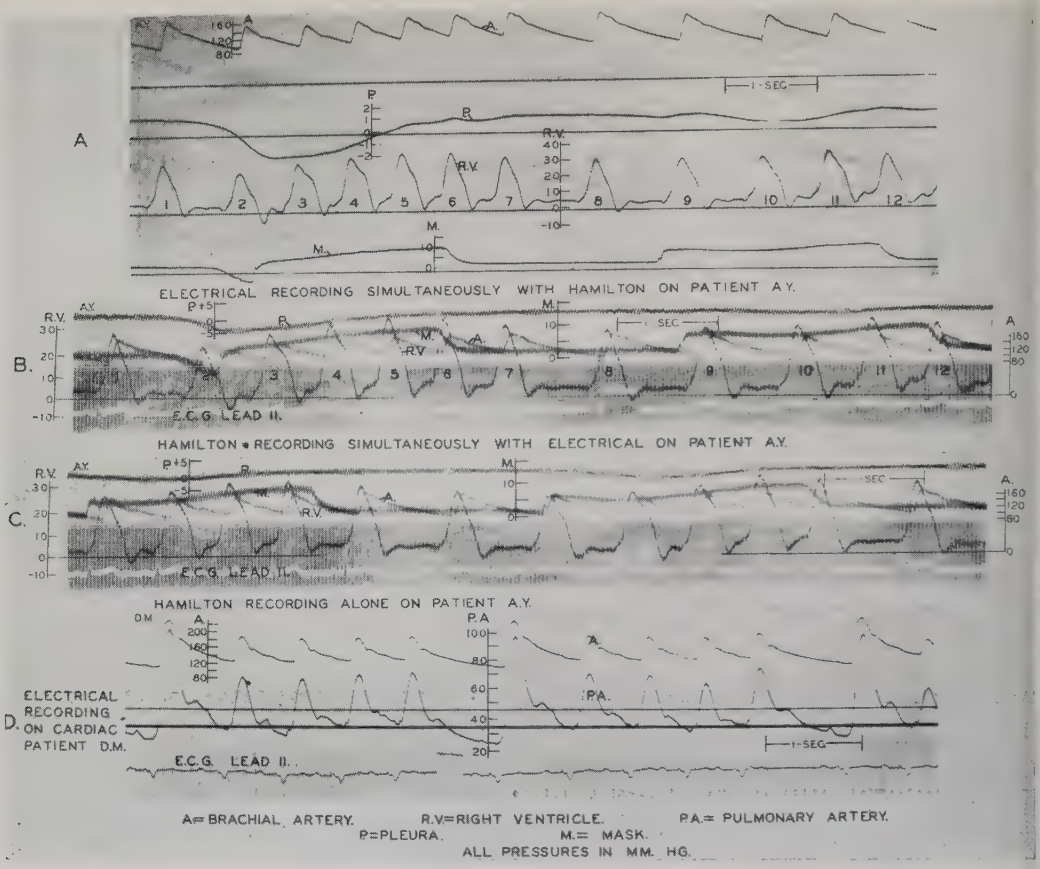

FIG. 1.

Electrical and Hamilton Pressure Recordings.

A. From above downward, brachial artery pressure, intra-pleural pressure, right ventricle pressure and face mask pressure. Patient breathing with an intermittent positive pressure respirator. Artificial pneumothorax previously present connected for pressure recording. Electrical recordings made simultaneously with the Hamilton given below and 12 consecutive corresponding heart beats shown, both arterial and right ventricle pressure pulse waves, the latter being numbered for ease in identification.

B. From above downward, intra-pleural pressure, face mask pressure, brachial artery pressure, right ventricle pressure and ECG lead II. Hamilton manometer recording made simultaneously with the electrical recording in A on the same patient.

C. Hamilton manometer recording alone on the same patient as above, but with the electrical apparatus disconnected. Tracing taken just previous to A and B shown above. Pressure tracings and ECG in the same order as in B above.

D. Electrical recording of brachial artery pressure (upper) and pulmonary artery (lower) with simultaneous ECG lead II in a cardiac patient with both systemic and pulmonary hypertension and a left bundle branch block.

diaphragm of varying thickness, depending on the pressure sensitivity required. The coils carrying a 1000 kc current on each side of the diaphragm of the capsule are balanced in the circuit of a bridge. When the 2 coils are properly balanced in the bridge a galvanometer connected through an amplifier for recording is set on the camera screen at a desired zero point. When pressure is exerted on one side of the capsule, a slight movement of the diaphragm occurs which alters the magnetic reluctance, creating an electrical imbalance of the bridge, which is amplified and the resulting galvanometer deflection recorded on an oscillograph type recording camera.

Electrical parts used:

1. Bridge (Balance type 3-103B Consol-

idated Engineering Corporation, Serial No. 3341).

2. Amplifier (Consolidated Engineering Amplifier, 4-channel type 1-106, Serial No. 3123, and Oscillator, carrier type 2-104B, Consolidated Engineering, Serial No. 2983).

3. Recording camera (Oscillograph, recording, type 5-101A, Consolidated Engineering, Serial No. 4775).

By changing the amplification a wide range of pressures could be measured without going off scale. Large deflections of the tracings could be secured for any pressure range encountered, and the amplification adjustment could be made quickly by turning a dial as needed. The calibrations for the electric recordings were linear for any given amplification setting and capsule, and remained constant from day to day.

To record blood pressure, the Clark Capsule was filled with airless mineral oil on the side attached to a 3-way stopcock and connected to an arterial needle or a cardiac catheter, in order to maintain a continuous fluid system, (sodium citrate or saline solutions in the capsule produced enough electrolysis so that the balance of the bridge became unstable). The bridge was balanced and the zero lines recorded at the beginning and end of each tracing taken. This procedure prevents errors in base line which might be made due to voltage drop, resistance changes, or drifts. Usually, when the base line did change due to one of the above factors, the deflection was of small magnitude. The Clark Capsule can be attached very closely to either the intraarterial needle or the cardiac catheter without need for lead tubing.

A Sanborn Cardiette was attached to the recording oscillograph using the amplifier of the Cardiette and one of the galvanometers in the oscillograph, for simultaneous recording of the ECG with the pressure tracings. Tests on conduction as measured by simultaneous recording of a pressure wave and ECG impulse showed a lag of .005-.01 second for the pressure wave as compared to the ECG. Similar tests on the Hamilton apparatus showed a lag of .01 second.[4]

TABLE I.

		Hamilton	Electrical
Arterial mm Hg	Systolic	166.8	166.1
	Diastolic	108.2	107.7
	Mean	133.0	133.0
Right Ventricle mm Hg	Systolic	30.0	30.8
	Diastolic	6.96	7.12
	Mean	11.5	12.0
Pleural mm Hg	Max.	+1.6	+1.4
	Min.	—3.0	—2.0
	Mean	+0.5	+0.6
Mask mm Hg	Max.	9.0	10.0
	Min.	2.0	2.0
	Mean	4.0	5.0

Pressure tracings (brachial artery and pulmonary artery) and ECG lead II taken simultaneously on a cardiac case with left bundle branch block and an irregular heart beat, are shown in Fig. 1, D.

The pressures were carefully measured beat by beat as recorded by the electrical and Hamilton methods simultaneously for 12 consecutive corresponding heart beats as shown in Fig. 1, A and B. The 2 systems were connected for simultaneous recordings by the use of a modified 3-way stopcock. A comparison was also made of pleural and mask pressures on the 2 systems. Inspection of Fig. 1, A and B, shows the general shape of all tracings on the 2 systems to be quite similar. The average quantitative values for the 2 systems are shown in Table I.

When the electrical system was cut off there was no significant change in the Hamilton recording either in shape of tracing or pressure values, as shown in Fig. 1, C.

It is beyond the scope of this report to discuss whether the blood pressure tracings obtained through the cardiac catheter and intraarterial needle are true records of pressure variation within the heart cavity and large arteries. Pressure tracings whether recorded with the electrical pickup or a Hamilton type may be modified by (a) artifacts due to movement of the tip of the catheter or of the catheter itself within the heart, (b) small volume changes in the catheter

[4] Cournand, A., Motley, H. L., Himmelstein, A., Dresdale, D., and Richards, D. W., Jr., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 148

due to its deformability, and (c) low damping frequency of the entire hydraulic system ranging from 12 to 40 cycles per second. The volume change in the Clark Capsule is slightly larger than in the Hamilton type of capsule, for a given pressure alteration. This might affect slightly the form of the pressure tracing. In so far as can be determined by comparison with the Hamilton, however, the Clark Pressure Capsule appears to be adequate for the purpose of measuring systolic, diastolic and mean blood pressures (Fig. 1).

The advantages of the electrical recording device are: (a) the indefinite use of the capsule membrane, (b) the constancy of the linear calibrations, (c) the ease of rapid adjustment of the amplification in order to meet any pressure range, as required by displacement of the catheter from right ventricle to the right auricle, etc., (d) the absence of lead tubing, (e) the more rapid recordings and (f) better control of the base lines. The disadvantages are: (a) the slightly larger volume change required in the Clark Pressure Capsule per unit of pressure change, as indicated above, (b) the high cost of the electrical amplification equipment as used in this study. The apparatus as described here for amplification has a much wider range than is necessary for blood pressure recordings. By further modification of this type apparatus, to meet the special needs of blood pressure recording, it should be possible to overcome these disadvantages.

Summary. 1. The Clark Capsule, an electrical pressure pickup device, has been found satisfactory for recording blood pressures from the right heart, pulmonary artery, systemic arteries, pleural and mask pressures in man, when used with a suitable amplification and recording device.

2. The tracings recorded with this apparatus compare closely with those obtained simultaneously with a Hamilton manometer system.

15757

Evidence that Virus of Herpes Simplex Does Not Cause Vincent's Angina of the Tonsil.*

ALEX J. STEIGMAN† AND THOMAS F. MCNAIR SCOTT.‡ (Introduced by W. Henle.)

The etiological role of the virus of Herpes Simplex in acute infectious gingivostomatitis has been established.[1] Not infrequently the Plaut-Vincent spirochete and the fusiform bacilli are also present, perhaps as secondary invaders, and prior to proof that the virus of Herpes Simplex was responsible, the spirochete-fusiform bacilli were regarded by some as causal. Thus, the disease was often called Vincent's gingivostomatitis.

It seemed worthwhile to investigate whether or not the virus of Herpes Simplex has any underlying causal role in Vincent's tonsillar angina. Each patient studied had a typical unilateral, craggy, yellowish ulcerated tonsil with membrane and foul odor. There was local adenopathy and low, if any, fever. Many Vincent's spirochetes and fusiform bacilli were seen on smear; no β-hemolytic streptococci or *C. diphtheriae* were cultured and no evidence of syphilis or infectious mononucleosis demonstrated.

Twelve young British soldiers (18 to 3[illegible] years of age) had the above typical picture

* Work done while serving with the American Red Cross Field Hospital Unit, Salisbury, England, 1941-1942.

† Department of Pediatrics, Temple University Medical School, Philadelphia, Pa.

‡ The Children's Hospital of Philadelphia (Department of Pediatrics, University of Pennsylvania), Philadelphia, Pa.

1 a. Dodd, Katherine, Buddingh, John, and Johnston, Leland, *Am. J. Dis. Child.*, 1939, **58**, 907; b. Burnet, F. M., and Williams, S. W., *M. J. Australia*, 1939, **1**, 637; c. Scott, T. F. McNair, and Steigman, Alex J., *J. A. M. A.*, 1941, **117**, 999; d. Black, W. C., *J. Pediat.*, 1942, **20**, 145.

Swabs and membrane of the tonsillar ulcer of each patient, together with his saliva, were rubbed onto the scarified cornea of a rabbit. None of the 12 rabbits developed the acute keratoconjunctivitis characteristic of an infection with the virus of Herpes Simplex,[1] or survived a challenge dose of this virus when given intracerebrally after an interval of about 3 weeks. Neutralization tests done with early and convalescent sera of these patients against a standard (HF) strain of herpes showed that those patients with absence of antibodies during the disease did not develop them in convalescence, while those with neutralizing antibodies in the acute serum did not show a rise of titer in the convalescent serum.

Technics. Those used for virus and for antibody studies were identical with standard methods as used by the authors in a previous study.[1]

Discussion. It has been shown that the common form of acute infectious gingivostomatitis as seen most frequently in children and occasionally in adults is due to a primary infection with the virus of Herpes Simplex. Since the etiology of this disease was in the past frequently ascribed to Vincent's organisms, although it is now known that it is caused by the virus of Herpes Simplex, a study was undertaken to determine whether the so-called Vincent's angina of the tonsil was also caused by this virus with the spirochete-fusiform combination acting as secondary invaders. In the 12 patients with Vincent's angina of the tonsil here studied, neither was the virus demonstrated in the membrane or saliva, nor did neutralizing antibodies against Herpes Simplex virus appear during convalescence. These facts, together with the striking effect of penicillin[2] in this disease, would negate any etiologic role of Herpes Simplex virus and is suggestive of a primary etiological role of the spirochetes themselves.

Summary. A study of 12 typical cases of Vincent's tonsillar angina showed that none of the cases were caused by an underlying infection with the virus of Herpes Simplex.

2 a. Denny, E. R., Stallenberger, P. H., and Pyle, H. D., *J. Oklahoma M. A.*, 1944, **37**, 193; b. Naegeli, F. C., and Morginson, W. J., *J. Am. Dental Assn.*, 1945, **32**, 1393; c. Shellenberger, P. L., Denny, E. R., and Pyle, H. D., *J. A. M. A.*, 1945, **128**, 706; d. Schwartz, B. M., *J. A. M. A.*, 1945, **128**, 704.

15758

Effects of Some Old and Proposed Anticonvulsants on the Threshold for Electrical Convulsions.

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Desirable improvements on diphenylhydantoin and other anticonvulsants in the treatment of epilepsy have stimulated the investigation of a large number of compounds with the hope of finding some agent which would prevent or mitigate epileptiform seizures without undesirable side effects.

Method. One of the methods used in these studies has been the production of epileptiform convulsions by passing an electrical current of varying amperage through the brains of animals. The threshold in milliamperes (m.a.) for each animal is determined before (control) and after the administration of the drug to be tested, the difference being considered a measure of the effectiveness of the drug as an anticonvulsant. The method has been described in detail by Spiegel,[1] Putnam and Merritt[2] and Tainter

1 Spiegel, E. A., *J. Lab. and Clin. Med.*, 1937, **22**, 1274.

2 Putnam, T. J., and Merritt, H. H., *Science*, 1937, **85**, 525.

and associates.[3] We used the technical arrangement described by Tainter and associates.[3]

In this arrangement, a high resistance stimulator and a 110-volt, 60-cycle, alternating current are used. In making a determination of cortical threshold, the transformer is set to give the voltage required for the desired m.a. of current, the electrodes (clamps) are clipped on the ears of the rats, and the stimulus is applied for exactly 10 seconds. In rabbits one electrode is applied to a bit in the mouth, and the other electrode to a small sponge-rubber pad resting on the clipped occipital skin moistened with saline solution. With currents below the threshold level there are generalized muscular contractions, synchronous with the current (tonic phase). At or above the threshold levels the tonic phase passes gradually into the clonic or epileptiform phase. The stimulation in rats is usually begun with a current of 6 m.a. (in rabbits, 14 m.a.), and is repeated at 5-minute intervals increasing the amount 2 m.a. each time until the threshold value is reached. This value remains practically unchanged for the purpose at hand, since it is reproducible within 1 to 2 m.a. when determinations are repeated at intervals of several days. Frequent, *e.g.*, consecutive daily, stimulations, however, tend to produce higher thresholds. Therefore, the animals are always allowed to rest 3 to 5 days before a drug is administered and the threshold redetermined. This procedure has been used routinely for several years in this department on hundreds of animals and with numerous drugs, and therefore, results, even with small groups of animals, are of significance in screening tests.

Employing this technic, a number of old agents and some proposed anticonvulsants were studied and the findings are reported in this paper. The test animals were chiefly adult white rats (total 185) with control thresholds ranging from 6 to 16 m.a. (average, 8 m.a.). A few rabbits (total 25 3 to a drug) were used for intravenous injections. Their thresholds usually were higher than in rats, ranging from 14 to 28 m.a. (average, 18 m.a.), although the results with the drugs used were similar to those obtained in rats and are described together. The drugs were given by various routes ½ to hour before redetermination of the threshold a change in which was not considered significant when, because of individual variations, it did not exceed the control threshold by more than 10%. When drugs were given gastrically, food was withdrawn from those animals one day previously. In the majority of tests, 5 rats were used for each drug and each dose, and occasionally up to 20 were used. The results obtained in rats with compounds which gave positive anticonvulsant activity are given in Table I.

Positive Agents. Demerol (isonipecaine meperidine or pethidine) and propazone were found to be effective only in excessive doses, as was methyl-N-hexylhydantoin (Table I). Of the other new hydantoins* studied only 2, namely, methyl-N-amylhydantoin and di-isobutyl-hydantoin, showed any promise and of these the former was effective only in doses which caused slight but definite ataxia. There was less ataxia with higher doses of di-isobutyl-hydantoin, but there was more variability, suggesting that cortical excitability did not decrease proportionately with increase in dosage of the drug. In general this drug seemed somewhat comparable to diphenylhydantoin.

Negative Agents. Tests were also made with the following drugs of the curare group: curare (2.5 to 5.0 mg per kg intramuscularly), β-erythroidine (10 to 400 mg per kg hypodermically; 50 to 800 mg per kg gastrically), dihydro-β-erythroidine (10 to 100 mg per kg hypodermically; 10 to 30 mg per kg intravenously) and quinine ethochloride (4 to 100 mg per kg hypodermically). These

[3] Tainter, M. L., Tainter, E. G., Lawrence, W. S., Neuru, E. N., Lackey, R. W., Ludueña, F. P., Kirtland, H. B., and Gonzales, R. I., *J. Pharm. and Exp. Therap.*, 1943, **79**, 42.

[4] Luton, F. H., Blalock, J., Baxter, J. H., Jr. and Stoughton, R. W., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 245.

* Obtained from Dr. Melville Sahyun, Frederic Stearns & Co., Detroit, Mich.

TABLE I.
Effect of Various Agents on Thresholds for Electrical Convulsions.

Compound	No. of rats	Dose, mg/kg	Route of administration*	Mean change in threshold, m.a.†	S.E. of mean, m.a.†	Avg incr. in threshold, %	Remarks
Demerol	20	5-25	H	+0.6	±0.27	7	No or slight motor depression
,,	4	50	H	+2.0	±0.55	28	Coma, fatal
Propazone	8	130-250	IP	+3.2	±0.53	43	Slight ataxia to coma
Diphenylhydantoin	5	40	G	0	±0.25	0	Asymptomatic
,,	5	40	IV	+1.0	±0.50	10	,,
,,	4	100	IV	+3.3	±0.60	41	Slight motor depression
3-indolyl methylene hydantoin	14	100-400	IM	+0.4	±0.42	40	Asymptomatic
Methyl-N-hexyl hydantoin	5‡	25	IV & IM	0	±0.32	0	,,
,, ,,	5	50	IV & IM	0	±0.44	0	Motor depression
,, ,,	5	100	IV & IM	+1.2	±0.82	15	,, ,,
,, ,,	10	200	IV & IM	+4.4	±1.45	55	Narcosis
Methyl-N-amyl hydantoin	5‡	50	H	0	±0	0	Asymptomatic
,, ,,	5	50	G	+1.6	±0.71	20	Slight ataxia
,, ,,	5	100	G	+2.4	±0.58	34	,, ,,
,, ,,	5	160	G	+4.4	±0.87	52	,, ,,
,, ,,	5	50	IM	+2.0	±0	25	Asymptomatic
,, ,,	4	80	IM	+3.0	±0.55	37	Slight ataxia
,, ,,	5	160	IM	+7.6	±0.70	95	Moderate ataxia
Di-isobutyl hydantoin	5	50	IM	+2.0	±0	28	Asymptomatic
,, ,,	5	100	IM	+1.2	±1.00	15	Slight ataxia
,, ,,	5	160	IM	+6.4	±0.75	80	,, ,,

* G—gastrically; H—hypodermically; IP—intraperitoneally; IM—intramuscularly; IV—intravenously.
† Milliamperes.
‡ Rabbits.

drugs did not raise the threshold, except in excessive doses which caused incomplete paralysis, or convulsions. With asymptomatic doses thresholds were unchanged or even decreased (possible excitatory action of anoxemia). These results did not support the claim that the curare group of drugs exert a central depressant action.[5]

The beneficial effects of certain dyes on patients, reported by different authors,[6] suggested studies of these agents, but trials with hematoxylin (1.5 g per kg), congo red and vital red (each 50 mg per kg intravenously), neutral red, phenol red and methyl red (each 50 mg per kg intraperitoneally) resulted in no change in threshold.

Other agents which were found to be without anticonvulsant activity were ammonium thiocyanate (0.1 g per kg gastrically), magnesium sulfate (0.7 g per kg intraperitoneally), theophylline sodium acetate (50 mg per kg hypodermically) and glutamic acid (0.1 to 0.2 g per kg hypodermically). Voluntary drinking of dilute acid (0.5% HCl) and of dilute alkali (1% $NaHCO_3$ or 0.01% NaOH) for 6 days, in place of drinking water, also proved ineffective.

A decrease in threshold of about 30% was obtained by depriving rats for 6 days of either water or food or both.

Summary. Over 400 tests were made in 210 animals with 23 different agents on the threshold for electrical convulsions, chiefly in white rats; a few in rabbits. Of 3 new hydantoins studied, di-isobutyl-hydantoin was the most promising, being somewhat comparable to sodium diphenylhydantoin in high doses. Among the ineffective agents were 4 drugs of the curare group, alleged central depressants, and 5 different dyes, including hematoxylin and vital red, alleged antiepileptic agents.

5 Culler, E. A., *Proc. Am. Physiol. Soc.*, 1939, p. 56; Feitelberg, S., and Pick, E. P., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 654; Harvey, A. M., and Masland, R. L., *J. Pharm. and Exp. Therap.*, 1941, **73**, 304; Pick, E. P., and Unna, K., *J. Pharm. and Exp. Therap.*, 1945, **83**, 59.

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15759

Isopropyl Alcohol, Other Ketogens, and Miscellaneous Agents on Thresholds for Electrical Convulsions and Diphenylhydantoin

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The efficacy but impracticability of a ketogenic diet in the treatment of patients subjected to epileptic seizures suggested the use of products of fat and carbohydrate metabolism and related compounds. With this in mind, the following experiments were carried out.

Method. Clonic (epileptiform) convulsions were produced by passing an electric current for 10 seconds through the brains of white rats, according to a technic described in detail by Tainter and associates.[1] A summary of the essential features of this method, adapted to rats, is given in a previous paper by Chu and Driver,[2] and need not be repeated here.

The control threshold, which ranged from 6 to 16 milliamperes (m.a.) (average, 8 m.a.)

1 Tainter, M. L., Tainter, E. G., Lawrence, W. S., Neuru, E. N., Lackey, R. W., Ludueña, F. P., Kirtland, H. B., and Gonzales, R. I., *J. Pharm. and Exp. Therap.*, 1943, **79**, 42.

2 Chu, W. C., and Driver, R. L., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 245.

TABLE I.
Isopropyl Alcohol and Ketogens on Thresholds for Electrical Convulsions and Diphenylhydantoin.

Compound*	No. of rats	Dose, mg/kg	Route of administration†	Hr before test	Mean change in threshold, m.a.§	S.E. of mean, m.a.§	Avg incr. in threshold, %
Acetone	5	1250	G	1	+ 4.4	±0.80	46
,,	5	,,	G	4	+ 1.2	±0.55	14
Acetophenone	3‡	,,	G	1	+ 7.3	±0.60	87
Ethyl acetoacetate	5	,,	G	1	+ 1.6	±0.75	15
Methyl alcohol	5	,,	G	1	+ 4.4	±0.82	41
Ethyl ,,	5	,,	G	1	+ 2.8	±0.80	26
Propyl ,,	5	,,	G	1	+ 3.2	±1.10	26
Isopropyl ,,	5	,,	G	1	+ 7.2	±0.85	82
,, ,,	5	,,	G	2	+ 4.8	±0.62	57
,, ,,	4	,,	G	4	+ 7.0	±1.40	75
,, bromide	5	,,	G	1	+ 2.8	±0.87	32
Diacetone alcohol	5	,,	G	1	+ 4.8	±0.51	55
,, glycol	5	,,	G	1	+ 6.0	±0.65	71
Sodium diphenylhydantoin	5	40	G	1	+ 0	±0.25	0
,, ,,	5	150	G	2	+ 1.2	±0.53	13
,, ,,	5	40	IV	1	+ 1.0	±0.50	10
,, ,,	4	100	IV	1	+ 3.3	±0.60	41
,, ,,	4	40	IM	2	+ 0	±0	0
,, ,,	5	100	IM	2	+ 1.0	±0.45	11
,, ,,	5	150	IM	2	+ 2.0	±0.70	23
,, ,,	5	40	G	1	+ 4.4	±0.42	40
+ ethyl alcohol		1250	G	1			
Sodium diphenylhydantoin	5	40	G	1	+12.2	±2.02	115
+ isopropyl alcohol		1250	G	1			

* An insoluble compound was administered as a suspension or emulsion.
† G—gastrically; IV—intravenously; IM—intramuscularly.
‡ 2 rats died.
§ Milliamperes.

was established for each rat at least 4 days before testing the effect of a compound. The substance was administered at a given time before the test, and the threshold redetermined. The difference in the thresholds so obtained was taken as a measure of the effectiveness of the medication, and, because of individual variations, had to exceed 10% to be considered significant. Food was withdrawn from all animals the night before the day of the test. The results (average % changes) including the standard errors (S.E.), with a number of positive agents and combinations are given in Table I. A total of 265 rats was used, in groups of 5 for each agent or combination in the majority of tests.

Isopropyl Alcohol and Other Ketogens. The results in Table I indicate that a number of compounds excelled sodium diphenylhydantoin as an anticonvulsant. Of particular interest were the compounds closely related to acetone in chemical structure. Of these, isopropyl alcohol, which exerted a tremendous anticonvulsant effect without ataxia or narcosis, proved to be one of the most promising. The dosage used, *i.e.*, 1250 mg per kg, was only about one-fourth the anesthetic dose. The animals were somewhat quieter than normals, but their reflexes and voluntary locomotion were preserved. Three months later all were alive and in good condition. The safeness of this agent for oral use has been reported by Harris.[3,4] Boughton,[5] comparing the relative toxicity of ethyl alcohol and isopropyl alcohol in rats given 5% solutions of these alcohols for 9 months, concluded that isopropyl was only slightly more toxic than ethyl alcohol. Lehman and Chase,[6] studying the acute and chronic tox-

[3] Harris, L. E., *Drug and Cosmetic Ind.*, 1944, **54**, 44.

[4] Harris, L. E., *J. Am. Pharmaceutical Assn., Pract. Pharm. Ed.*, 1944, **5**, 38.

[5] Boughton, L. L., *J. Am. Pharmaceutical Assn.*, 1944, **33**, 111.

[6] Lehman, A. J., and Chase, H. F., *J. Lab. and Clin. Med.*, 1944, **29**, 561.

icity of isopropyl alcohol given to, and drunk voluntarily by rats reported no evidence of delayed toxic effects and no suggestion of harmful intermediate products, while the acute effects were similar to those of ethyl alcohol. These reports are typical of many on the toxicity of isopropyl alcohol. Its superiority to ethyl alcohol as an anticonvulsant is, according to biochemical evidence, probably due to its partial transformation to acetone in the body.[7–11] This may also be the explanation of the beneficial effects of a ketogenic diet in epileptics, although, of course, the ultimate mechanism remains unsolved.

Beta-hydroxy-butyric acid and acetoacetic acid were not tried, but diacetone alcohol* (4-hydroxy-2-keto-4-methylpentane) and diacetone glycol* (2,4-dihydroxy-4-methyl pentane) were quite effective, while ethyl acetoacetate was slightly so. The difference in activity between isopropyl alcohol and acetone was possibly due to differences in rate of absorption and excretion.[7] Acetone, after 4 hours, caused only a small increase in the threshold, presumably because this agent was rapidly eliminated from the body due to its volatility. The positive effect of isoprophyl bromide could conceivably have been due to 2 things: (1) liberation of bromide and (2) formation of acetone. Acetophenone was quite effective but was too toxic because it caused fatalities. Aside from acetophenone, in this group of ketogens, only diacetone alcohol caused demonstrable ataxia.

Isopropyl Alcohol and Diphenylhydantoin Combined. Since the available evidence favored isopropyl alcohol as the least hazardous and most desirable of all the agents tested, it was tried in combination with diphenyl hydantoin. A greater effect than summation of activity, perhaps a sensitization, was obtained by combining sodium diphenylhydantoin in doses of 40 mg per kg with isopropyl alcohol, given gastrically, because diphenyl hydantoin itself caused no significant increase in threshold, while the combination of the 2 drugs exceeded considerably the depression caused by isopropyl alcohol itself (Table I). Ethyl alcohol, combined the same way, only moderately exceeded the threshold for the alcohol itself, thus indicating that isopropyl alcohol was more specific in this respect. Here again, raising of the cortical threshold with the combinations of isopropyl alcohol and diphenylhydantoin occurred without demonstrable evidence of ataxia or narcosis and all the animals recovered. It is suggested that the isopropyl group (or isopropanol), in view of its relative nontoxicity and close correlation with the ketogenic mechanism, would seem to offer possibilities for developing new, or improving old, anti-epileptic agents.

Ineffective Agents. The following compounds were found to be ineffective: the sugars—glucose, fructose, galactose, sucrose and triose (each 1.5 g per kg); the organic acids—lactic, pyruvic, acetic, propionic, butyric, palmitic, citric, malic, succinic, malonic and fumaric (each 0.5 g per kg), iodoacetic acid (0.02 g per kg); the alcohols—butyl, isobutyl and isoamyl (each 1.25 g per kg); insulin (2.5 units per kg), isopropyl ether and ethyl acetate (each 1.25 g per kg). All these agents were given gastrically except insulin and iodoacetic acid, which were injected hypodermically.

Summary. The majority of certain compounds tried, and related in chemical structure to acetone, particularly isopropyl alcohol, were found to raise considerably the threshold for electrical convulsions in rats. Isopropyl alcohol increased the cortical depressant efficiency of diphenylhydantoin without demonstrable motor depression or narcosis. Some other alcohols and certain sugars and organic acids were ineffective. The possible significance of the isopropyl group

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8 Kemal, H., *Z. physiol. Chem.*, 1937, **246**, 59.

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10 Morris, H. J., and Lightbody, H. D., *J. Ind. Hyg. and Tox.*, 1938, **20**, 428.

11 Lehman, A. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 232.

* These and some other related agents were supplied by the Shell Development Co., Emeryville, Calif.

(or isopropanol) for benefits from ketogenic agents in epilepsy and for developing new or improving old antiepileptic agents is discussed.

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Hydropic Changes in Pancreatic Ductules and Islets in Alloxan Diabetes in the Rabbit.*

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(Introduced by J. B. Collip.)
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Several investigators have described hydropic degeneration of the pancreatic islets and ductules in dogs rendered diabetic by partial pancreatectomy[1,2] and by anterior pituitary extracts.[3-5] In dogs made diabetic by alloxan, hydropic degeneration of islet cells has not been observed even in the presence of extreme vacuolation of the epithelium of the intralobular pancreatic ducts.[6] Hydropic changes have been observed in the islets of cats that had become diabetic following partial pancreatectomy[2] or treatment with anterior pituitary extract[7] but the pancreatic ductules were not affected. While such alterations in occasional islet cells have been described in rabbits treated with alloxan[8,9] the duct epithelium is said to remain normal.[10] The only description of alterations in the epithelium of pancreatic ductules in diabetic rabbits is that of Ogilvie[11] who found slight vacuolation of the lining cells of "newly formed" intralobular ducts in 1 of 28 rabbits treated with anterior pituitary extract. It is interesting, therefore, to report a high incidence of moderate to extreme hydropic degeneration of both the ductules and islets of the rabbit's pancreas following diabetes of long duration induced by alloxan.

Materials and Methods. Each of 56 white domestic rabbits obtained from several different dealers received intravenously 200 mg of alloxan (Eastman) per kg of body weight in a 5% aqueous solution. They were treated with protamine zinc insulin and glucose for a period of not more than 14 days following alloxan injection. In all of these animals and in a control group of 26 untreated rabbits, repeated determinations were made of fasting blood sugar and urinary sugar and acetone. Surviving animals of the experimental group were killed with corresponding control animals at various intervals up to one year after injection of alloxan.

Observations. Of the 56 rabbits treated with alloxan, 53 became persistently diabetic

* This work was assisted by grants-in-aid from the Cooper Fund of the Faculty of Medicine, McGill University, and from the National Research Council, Canada.

† Medical Research Fellow of the National Research Council, Canada.

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11 Ogilvie, R. F., *J. Path. and Bact.*, 1944, **56**, 225.

TABLE I.
Summary of Experimental Data.

Duration of diabetes in mo.	No. of animals	Grade of Hydropic Degeneration: Islets 0	Islets 1	Islets 2	Islets 3	Islets 4	Ductules 0	Ductules 1	Ductules 2	Ductules 3	Ductules 4	Mean of avg blood sugar mg per 100 cc
0-1	19	19	—	—	—	—	19	—	—	—	—	428
1-2	8	1	2	4	1	—	1	3	2	—	2	388
2-3	4	1*	1	2	—	—	—	—	2	—	2	379
3-4	9	1†	2	4	—	2	1†	—	—	3	5	422
4-5	4	—	—	2	2	—	—	—	1	1	2	432
5-6	2	1‡	—	1	—	—	1‡	—	1	—	—	310
6-7	2	—	—	1	1	—	—	—	1	—	1	337
7-8	2	—	—	2	—	—	—	—	1	1	—	444
8-9	2	—	—	1	1	—	—	—	1	1	—	401
12	1	—	—	—	1	—	—	—	—	1	—	256
Totals	53	23	5	17	6	2	22	3	9	7	12	
Control	26	26	—	—	—	—	26	—	—	—	—	105
Alloxan resistant	3	3	—	—	—	—	3	—	—	—	—	123

* No islets identified.
† Blood sugar average 280 mg per 100 cc.
‡ " " " 177 " " " "

as indicated by persistent polyuria, glycosuria, polydypsia, polyphagia, loss of weight and hyperglycemia. Three animals proved to be resistant to the diabetogenic action of alloxan. The latter were killed and examined at intervals of 4, 6 and 11 months respectively after the administration of alloxan.

In Table I are shown the results of the experiments with particular reference to the incidence of hydropic degeneration of the islet and ductular epithelium and the duration of the experiments in which such changes occurred, the duration being calculated from the day of injection of alloxan. The degree and extent of hydropic change was graded histologically on a scale of 0 to 4.

In histological sections of the pancreas, the islets of Langerhans showed varying degrees of reduction in size from animal to animal with from very slight decrease in number to almost total absence of identifiable islets. The hydropic islet lesion consisted of varying degrees of vacuolation of the cytoplasm of the affected cells. The nucleus was centrally located and appeared normal but the cytoplasm in severely affected cells was almost totally replaced and the cell membrane distended by a single vacuole in which no content could be fixed or stained by routine histological methods (Fig. 1C). The other islet cells that were not affected were readily stained by routine methods and were shown to be almost exclusively alpha cells by the Gomori granule stain. These cells in many islets appeared to be considerably more numerous than could be accounted for by mere reduction in size of the islets from destruction of beta cells and consequent condensation of surviving alpha cells. Although this appearance suggested proliferation of alpha cells, mitotic figures were not found in any of the cells of the islets of Langerhans.

The affected intralobular ductules were only those that are normally lined by cuboidal epithelium. They were found to arise from normal ducts of larger size lined by columnar epithelium and in their finer ramifications to approach, and on occasion even to enter, hydropic islets (Fig. 1A). The earliest noticeable change was a swelling of the cuboidal epithelium that rendered the minute ductules more conspicuous than usual. This was followed by dissolution of the cytoplasm of varying degree up to complete disappearance of the cytoplasm, the place of which was taken by a large clear space bounded by the distended cell membrane (Fig. 1A and B). The centrally placed nucleus appeared slightly smaller and more deeply staining than

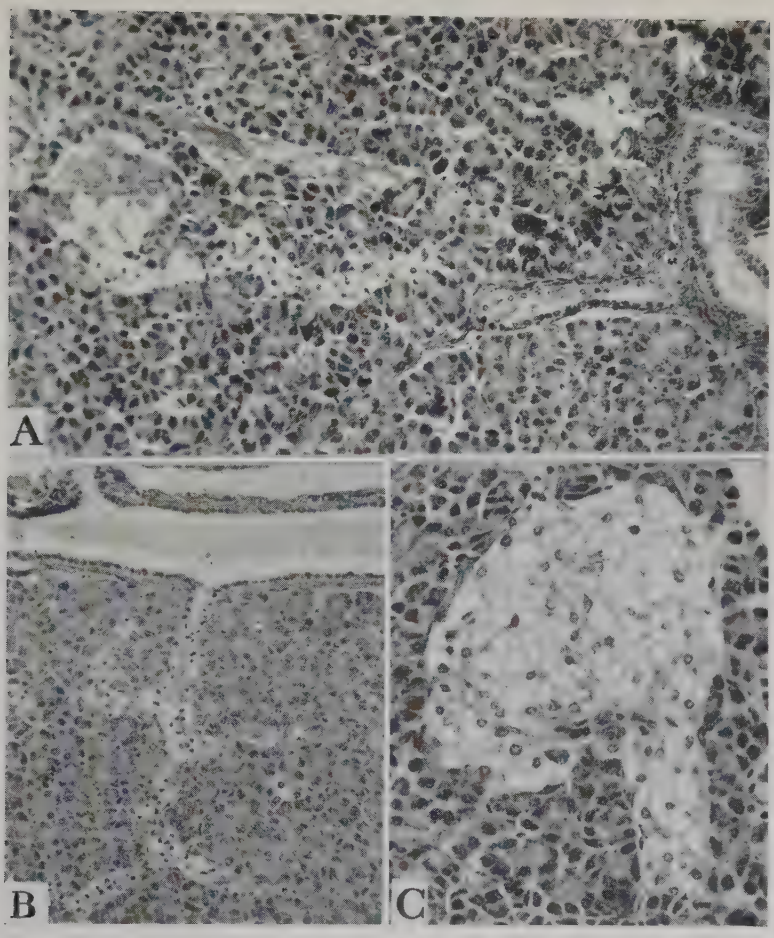

FIG. 1.
Photomicrographs of Sections of Pancreas Stained with Haematoxylin and Eosin.

A. At the right is a duct lined by columnar epithelium of normal appearance. A smaller duct arising from it is lined by cuboidal epithelium, the cells of which show a variable degree of hydropic change. From this latter duct an intralobular ductule exhibiting extreme hydropic degeneration comes into continuity with an islet composed of clear distended hydropic cells and a group of normally stained alpha cells. × 165.

B. A branching intralobular ductule presenting extreme hydropic degeneration of its lining epithelium is shown arising directly from a major duct, above, of which the columnar epithelium is normal in appearance. Note the compact hyperchromatic nuclei of the hydropic cells. × 135.

C. This unusually large islet of Langerhans illustrates the typical appearance of the hydropic changes observed in islet cells. The islet is exceptional in that it contains in the plane of section only two identifiable alpha cells which lie together toward its lower margin. × 200.

normal. The content of the vacuoles, as in the case of the hydropic islet cells, could not be fixed or stained and in both instances special staining to demonstrate glycogen, fat or mucin failed to reveal the presence of any of these substances. The lumina of many affected ductules were dilated by a small content of acidophilic, homogeneous material. It was found to be impossible in some cases to determine morphologically the islet or ductule origin of many groups of hydropic cells, especially where there was extreme hydropic degeneration in the absence of recognizable alpha cells. It is to

be noted, however, that, with one exception, islet and ductule lesions were found to occur concomitantly. This exception was accounted for by the absence of positively identifiable islets in the sections of pancreas from this one animal.

In addition, a similar hydropic change was observed in isolated single cells, and small groups of cells scattered throughout the acinar tissue. It was impossible to determine morphologically the origin of these cells. However, it was apparent that they were not hydropic acinar cells.

Discussion. Although our experiments were not designed to permit a detailed analysis of the etiological factors concerned in the production of hydropic degeneration of islet and ductule cells of the pancreas, nevertheless, it is apparent that both time and a diabetic state were essential. Table I shows that hydropic degeneration was not found in any of the animals that were diabetic for less than one month. The earliest occurrence of the lesion was found at 45 days, and it was invariably present after 90 days providing that the average fasting blood sugar level during this period had been 303 mg per 100 cc, or higher. One diabetic rabbit with an average blood sugar level of 256 mg showed moderate hydropic degeneration of islet and ductule cells after 12 months. A statistical comparison of the mean of the average blood sugar content of the animals showing hydropic changes of Grades 1 and 2 with that of animals showing Grades 3 and 4 indicated that there was no significant difference between the blood sugar levels of the 2 groups. The degree of hydropic degeneration, therefore, was found to be independent of the degree of hyperglycemia. The absence of hydropic pancreatic lesions in the 3 animals that were found to be resistant to the diabetogenic action of alloxan and in the 2 animals that exhibited relatively mild diabetes, indicated that alloxan *per se* was not the etiological factor concerned.

In the dog and cat, hydropic degeneration of the previously normal beta cells of the islets of Langerhans, whether produced by partial pancreatectomy[1,2] or by the administration of anterior pituitary extracts,[3–5,7] is followed by disintegration of the hydropi cells and a corresponding reduction in siz of the islets. This stage of atrophy of islet is reached after 4 to 6 weeks of severe dia betes in the dog,[3–5] but only after 3 to months in the cat.[7] In contrast to thes relatively short periods, it is interesting t note that in alloxan diabetes in the rabbi the hydropic degeneration of the pancreati islets persists without histological evidence o any disintegration of the affected cells i association with more or less severe diabete lasting for periods up to one year. How ever, in this case it is not by any mean certain that the hydropic cells are altere beta cells and, indeed, this would appear t be rather doubtful since most, if not al of the beta cells of the islets were pre sumably destroyed by the initial administra tion of a rather large diabetogenic dose o alloxan. In the islets of Langerhans of vari ous species studied histologically 5 days o more after the administration of alloxan sev eral investigators have mentioned the pres ence of a few indifferent cells with clear, non granular cytoplasm in addition to the sur viving alpha cells.[6,8,12–14] If in our experi ments the numerous vacuolated islet cell arose from the proliferation and subsequen hydropic degeneration of such indifferen cells or of undifferentiated ductular epitheli um, these cells might be expected to be n more susceptible to ultimate destruction tha the hydropic epithelium of the ductules them selves.

Nevertheless, preliminary experiments hav demonstrated by repeated biopsy of the pan creas that the hydropic condition of both islet and ductular epithelium is reversible by adequate treatment of the diabetic stat with insulin. Many of the reversed islet cells in the few histological sections of pancreas that we have thus far studied are indifferent cells with clear, nongranular cytoplasm, bu others possess a finely granular cytoplasm in

12 Gomori, G., and Goldner, M. G., PROC. SOC EXP. BIOL. AND MED., 1943, **51**, 287.

13 Hard, W. L., and Carr, C. J., PROC. SOC. EXP BIOL. AND MED., 1944, **55**, 214.

14 Duff, G. L., and Starr, H., PROC. SOC. EXP BIOL. AND MED., 1944, **57**, 280.

distinguishable from that of the normal beta cells of the islets of normal rabbits. Still other cells present a patchy granularity of the same type and of varying extent, so that all stages of transition from the nongranular cells to cells with a full complement of granular cytoplasm are observed. It is possible that such a transition from nongranular to granular cells of beta type may actually occur under the influence of insulin therapy. This, together with the appearance of proliferation of both of these types of cells, suggests the possibility of improvement or even complete restoration of islet function in relation to carbohydrate metabolism by suitable manipulation of insulin therapy in alloxan diabetes in the rabbit.

Summary. Moderate to extreme hydropic degeneration of the pancreatic ductules and islets was observed in rabbits rendered diabetic by alloxan when the diabetic state had persisted for several months. Such changes have not hitherto been described in alloxan diabetes in the rabbit. The earliest appearance of these alterations was at the end of 45 days after the injection of alloxan and hydropic changes were never absent after 90 days providing that the average fasting blood sugar level during the experiment had been 303 mg per 100 cc, or higher. The hydropic state of the ductules and islets persisted without histological evidence of any further change in association with more or less severe diabetes lasting for periods up to one year. The alpha cells of the islets remained histologically normal but appeared to be increased in number. Preliminary observations demonstrated that the hydropic degeneration of both ductules and islets is reversible by adequate treatment of the diabetic state with insulin. The reversed islet cells appeared to be unduly numerous suggesting proliferation. They were made up in part of indifferent nongranular cells and in part of cells exhibiting varying degrees of granularity of the cytoplasm of beta type. Those with a full complement of granular cytoplasm were indistinguishable in appearance from the beta cells of the islets of Langerhans in the normal rabbit.

15761

Antibacterial Action of N-Alkyl *p*-Aminobenzoic Acid Derivatives.*

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Yeomans *et al.*,[1] have recently demonstrated the beneficial therapeutic effect of *p*-aminobenzoic acid in louse-borne typhus fever, and several investigators since then have shown its effectiveness in experimental rickettsial infections.[2-5] In a study on the mode of action of sulfonamides, Wyss, Rubin and Strandskov[6] synthesized several ring-substituted *p*-aminobenzoic acid derivatives and tested them for antibacterial action. They found that the 2-Cl, 3-Cl, 2-NH_2, 3-NH_2, and 3-F derivatives of *p*-aminobenzoic acid displayed varying degrees of bacteriostatic action which could be reversed by the addi-

* The authors wish to express their appreciation to Dr. A. R. Surrey for making available the compounds used in this study. His methods of synthesis appear elsewhere.[10] We are also indebted to Mrs. Beatrice Bass for technical assistance.

1 Yeomans, A., Snyder, J. C., Murray, E. S., Zarafonetis, C. J. D., and Ecke, R. S., *J. A. M. A.*, 1944, **126**, 349.

2 Anigstein, L., and Bader, M. N., *Science*, 1945, **101**, 591.

3 Hamilton, H. L., Plotz, H., and Smadel, J. E., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 255.

4 Hamilton, H. L., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 220.

5 Murray, E. S., Zarafonetis, C. J. D., and Snyder, J. C., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 80.

6 Wyss, O., Rubin, M., and Strandskov, F. B., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 155.

tion of *p*-aminobenzoic acid itself. At pH 7.0 the most active derivative (2-Cl) had a bacteriostatic value equal to sulfapyridine. Hirsch[7] found *p*-aminobenzamide to be as active as sulfanilamide, and postulated that a new group of substances with chemotherapeutic effects toward bacterial infections might be found among the derivatives of *p*-aminobenzamide.

The present authors investigated the effects upon sulfonamides of a group of N-substituted *p*-aminobenzoic acid derivatives and local anesthetics derived from *p*-aminobenzoic acid.[8,9] During these studies it was noted that several of the compounds, notably the methyl *p*-alkylaminobenzoates, gave evidence of possessing considerable antibacterial activity. It seemed appropriate, therefore, to study more completely the general antibacterial properties of the various alkyl-substituted *p*-aminobenzoic acids.

Methods of Antibacterial Tests. The medium employed throughout these tests was trypticase soy broth (B.B.L.). This medium was found to support excellent growth of all test organisms without the addition of serum or other growth-promoting factors. The organisms employed were as follows: Pneumococcus type II, *Streptococcus pyogenes* (C203), *Staphylococcus aureus, Haemophilus ducreyi, Pasteurella pestis, Eberthella typhi, Vibrio cholerae,* and *Mycobacterium avium.*

A series of dilutions of the *p*-aminobenzoic acid derivatives ranging from 1:1000 to 1:64,000 was prepared directly in the broth. Owing to the relative insolubility of several of the compounds in water, the initial dilutions were made by dissolving the compounds in a small amount of alcohol followed by the addition of broth. Controls containing an equivalent amount of alcohol had no visible effect upon the growth of the organisms. Tubes containing 5 cc of each dilution were autoclaved at 10 lb for 10 minutes, and after cooling 0.05 cc of a 1:100 dilution of a 24-hour broth culture of test organism was added to each series. The tubes were incubated at 37°C and examined after 24 hours for visible growth (48 hours in tests employing the pneumococcus, streptococcus and *P. pestis*). Bacteriostasis was recorded for those tubes showing no growth or growth less than half that of the drug-free broth control at the initial observation time. All tubes containing no growth after 72 hours at 37°C were subcultured by transferring 3 4-mm loopfuls to 10 cc of fresh trypticase soy broth. Failure of growth to appear in the subculture tube after 72 hours' incubation was taken as evidence of a bactericidal action by the drug in the original medication tube. Results of these tests are presented in Table I.

Results. It is apparent that the antibacterial activity of *p*-aminobenzoic acid is slightly increased by the substitution of an ethyl or butyl group in the N-position. In the series of methyl *p*-alkylaminobenzoates and ethyl *p*-alkylaminobenzoates maximum activity is found in the N-propyl-substituted compounds. In this case the N-ethyl and N-*n*-butyl derivatives are about equal in action which is somewhat less than the N-propyl compounds. The series of benzamides display a direct correlation between chain length of the N-substituent and degree of antibacterial action. The lowest degree of activity is exhibited by the ethyl derivative with proportionately increasing activity being found in the propyl and *n*-butyl derivatives.

Antagonism by p-aminobenzoic acid. Inasmuch as Hirsch[7] noted that *p*-aminobenzamide was antagonized by the presence of *p*-aminobenzoic acid, and we showed that the *p*-alkylaminobenzamides did not antagonize sulfonamides[8] it appeared worthwhile to study the effect of *p*-aminobenzoic acid upon the *p*-alkylaminobenzamides, and upon diethylaminoethyl - *p-n* - butylaminobenzoate hydrochloride (the *n*-butyl derivative of procaine hydrochloride, U.S.P.). The latter compound had also been found to have none of the sulfonamide antagonizing properties of procaine itself.[9]

Accordingly, diethylaminoethyl-*p-n*-butyl-

[7] Hirsch, J., *Science,* 1942, **96**, 139.

[8] Goetchius, G. R., and Lawrence, C. A., *J. Bact.,* 1944, **48**, 683.

[9] Lawrence, C. A., and Goetchius, G. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 180.

[10] Surrey, A. R., and Hammer, H. F., *J. Am. Chem. Soc.,* 1944, **66**, 2127.

TABLE I. Antibacterial Action of *p*-Aminobenzoic Acid Derivatives.

Figures in each left-hand column represent highest dilution × 1000 exhibiting bacteriostatic action, *i.e.*, 2 = 1:2000, etc. Figures preceded by symbol < denotes highest concentration tested (1:1,000) failed to show evidence of bacteriostasis. Figures followed by asterisk (*) represents highest dilution exhibiting bactericidal activity.

Compound	Pneumococcus type II	*Streptococcus pyogenes*	*Staphylococcus aureus*	*Haemophilus ducreyi*	*Pasteurella pestis*	*Eberthella typhi*	*Vibrio cholerae*	*Mycobacterium avium*
p-Aminobenzoic acid	<1	<1	<1	1	1	<1	<1	<1
p-Ethylaminobenzoic acid	2 1*	<1	<1	1	2	<1	<1	<1
p-Butylaminobenzoic acid	2 1*	1	<1	1	4 2*	<1	2 1*	<1
Diethylamino ethyl *p*-ethylamino benzoate. HCl	4	<1	<1	<1	<1	<1	<1	<1
Diethylamino ethyl-*p*-*n*-butylamino benzoate. HCl	8 4*	4 4*	1 1*	2 2*	2 2*	1	2 1*	1
Methyl *p*-ethylamino benzoate	2	1	<1	1	2	<1	<1	<1
Methyl *p*-propylamino benzoate	8 1*	16 8*	<1	8 2*	8 8*	<1	4 1*	<1
Methyl *p*-*n*-butylamino benzoate	2 1*	1	<1	2	2	<1	<1	<1
Ethyl *p*-ethylamino benzoate	16 1*	4 1*	<1	4	8 8*	<1	4 1*	<1
Ethyl *p*-propylamino benzoate	32 1*	16	<1	4 1*	16 2*	1	2	<1
Ethyl *p*-n-butylamino benzoate	16	4	<1	32 1*	4 2*	1	1	1
p-Ethylamino benzamide	2	<1	<1	1	1	<1	<1	<1
p-propylamino benzamide	4	2	<1	2	1 1*	1	1 1*	<1
p-*n*-Butylamino benzamide	8 2*	4 2*	1	4 1*	2 2*	1 1*	2 1*	1

aminobenzoate hydrochloride and *p*-*n*-butylaminobenzamide were dissolved in 1% concentration directly into trypticase soy broth containing 10^{-3}, 2×10^{-4}, 10^{-4}, and 2×10^{-5} dilutions of *p*-aminobenzoic acid. The initial concentrations of the 2 test compounds were then diluted serially up to and including dilutions of 1:12,800. The compounds were also diluted in a similar manner in trypticase soy broth containing no *p*-aminobenzoic acid for control titrations. The tubes were autoclaved at 10 pounds for 10 minutes, and after cooling were inoculated with a 4-mm loopful of a 24-hour broth culture of *Streptococcus pyogenes* (C203). The inoculated tubes were incubated as before and the bacteriostatic and bactericidal end-points recorded.

Results. The data obtained in this study revealed that *p*-aminobenzoic acid does not antagonize the antistreptococcal activity of either diethylaminoethyl-*p*-*n*-butylaminobenzoate hydrochloride or *p*-*n*-butylaminobenzamide. It would appear from these findings, therefore, that N-alkylation of these *p*-aminobenzoic acid derivatives not only nullifies their antagonistic action upon sulfonamides, but also gives their own antibacterial activity an "immunity" to antagonism by *p*-aminobenzoic acid.

Summary. 1. The bacteriostatic and bactericidal activity of a series of N-substituted *p*-aminobenzoic acid derivatives have been determined.

2. Among the acids the order of decreasing activity is as follows: *p*-butylaminobenzoic acid > *p*-ethylaminobenzoic acid > *p*-aminobenzoic acid.

3. Of 2 procaine hydrochloride (U.S.P.) derivatives, the N-butyl derivative displays greater antibacterial action than the N-ethyl.

4. Among the methyl and ethyl esters of *p*-aminobenzoic acid, the N-propyl derivatives display more activity than the N-ethyl or N-butyl compounds. The latter 2 were approximately equal in respect to antibacterial action.

5. The order of decreasing activity among the *p*-alkylaminobenzamides was: butyl > propyl > ethyl.

6. Diethylaminoethyl-*p*-*n*-butylaminobenzate hydrochloride and *p*-*n*-butylaminobenzamide were not antagonized by the presence of *p*-aminobenzoic acid when tested in trypticase soy broth against *Streptococcus pyogenes* (C203).

15762

Action of Certain Carbohydrates on the Reaction of *Eberthella typhosa* with Antibody O.

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In a previous publication, Olitzki, Shelubsky and Hestrin[1] reported that certain carbohydrates promote the pathogenicity of *E. typhosa*. Since Gram-negative microorganisms are normally eliminated from the peritoneal cavity in the mouse mainly by bacteriolysis a short time after the onset of infection,[2] the possibility that the carbohydrates, which alter pathogenicity might also interfere with a bacteria-antibody reaction seemed to deserve consideration. The influence of the carbohydrates previously employed in tests of pathogenicity on the agglutination of typhoid strain 0901 by its O-antibody, the presence of which in antisera parallel the bactericidal activity,[3] has accordingly been examined. The results of the agglutination tests are shown in Table I.

Table I shows that the carbohydrates used for testing, *viz.* dextran, levan, mucin and pectin, markedly inhibit agglutination. The results were the same whether bacteria were added to the serum-carbohydrate mixture immediately after its preparation or after it had been incubated for 4 hours at 37°C.

The mechanism of the inhibition could be a serological reaction between the test carbohydrates and the typhoid antibody. We have not, however, been able to detect a visible precipitation reaction when the carbohydrates were mixed with anti-typhoid serum. In order to ascertain whether the inhibition is produced by interference on the part of the carbohydrates with fixation of antibodies by the bacterium, or by an intervention in the second stage of the agglutination reaction, we measured the effect of the more active of the inhibitors (dextran 2%, levan 2%, pectin 2%, and mucin 5%) on antibody-nitrogen uptake.

The carbohydrate solutions were sharply centrifuged to remove all insoluble particles. Heat-killed and washed bacteria sediment containing 0.419 mg nitrogen was then suspended in 2.0 cc of the solutions. After the selected carbohydrates and bacteria had been in contact for 2 hours at 37°C, the immune serum was added and the mixture allowed to stay for 24 hours in the ice box. The bacteria were then removed and washed and the N-uptake determined by a Micro-Kjeldahl method. In another series of experiments the serum was left in contact with an inhibitor for 2 hours at 37°C. Examination of this mixture failed to reveal any visible precipitation. Bacterial sediment containing 0.260 mg nitrogen was then suspended in the mixture. The suspension was left for 24 hours in the ice box. The bacteria were then removed and examined as in the previous series. Both series of experiments gave similar results. The control test without the addition of inhibitor showed that the bacteria are normally able to remove in 24 hours 0.033 mg N from 0.1 ml serum and 0.061 mg N from 0.2 ml serum. In the pres-

[1] Olitzki, L., Shelubsky, M., and Hestrin, S., in press.

[2] Olitzki, L., and Koch, P. K., *J. Immunol.*, 1945, **50**, 229.

[3] Felix, A., and Olitzki, L., *J. Immunol.*, 1926, **11**, 31.

TABLE I.
Influence of Pathogenizing Substances on Agglutination of the 0901 Strain by Anti-O Serum (Titer 1:10,000).

Substance tested	Concentration, %	End of the agglutination titer
Dextran (*L. mesenteroides*)	0.1-0.25	1:10,000
	0.5-1.0	1: 1,000
	2.0	negative at 1:100
Levan (*A. levanicum*)	0.1-0.5	1:10,000
	1.0	1: 5,000
	2.0	negative at 1:100
Mucin	1.0	1:10,000
	2.0	1: 2,000
	5.0	negative at 1:100
Pectin	0.1-0.2	1:10,000
	0.5	1: 1,000
	1.0	1: 100
	2.0	negative at 1:100
Glycogen	0.5	1:10,000
	1.0	1: 2,000
	2.0	1: 1,000
Starch	1.0-2.0	1:10,000
	5.0	1: 200
Inulin, gum acacia, cellulose (cotton)	0.1-5.0	1:10,000

ence of any of the inhibiting substances (dextran, levan, mucin, pectin), however, no measurable quantities of antibody N were taken up by the cells. These observations accord with the findings of Keefer and Spink[4] concerning the effect of mucin on the bacteriolysis of gonococci by whole blood and immune serum.

[4] Keefer, C. S., and Spink, W. W., *J. Clin. Invest.*, 1938, **17**, 23.

Summary. The union of O-antibody by *E. typhosa* is completely inhibited by dextran, levan, and pectin at 2% concentration and by mucin at 5% concentration. Reduction of agglutination titer occurs in the presence of starch (5%) and glycogen (1-2%). Inulin, gum acacia, and acid-degraded cotton cellulose in 5% concentration are without this effect.

15763

Influence of Nitrogen Mustards on the Antibody Response.*

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The absence of infection in the leucopenic phase following nitrogen mustard therapy of lymphomas suggests that some specific protective mechanism persisted despite the demonstrable widespread damage to the hemopoietic system. The role of lymphocyte in the antibody response[1,2] suggests the lym-

* This study was supported in part by a grant from the Committee on Growth, American Cancer Society.

[1] Ehrich, W. E., and Harris, T. N., *J. Exp. Med.*, 1942, **76**, 335.

phocytotoxic nitrogen mustard compounds may influence the antibody reaction.

Method. To elucidate this relationship, the response to typhoid vaccine 1 cc intramuscularly, in a single dose and in 3 weekly doses was followed at 3-7-day intervals by agglutination titration with dilutions of 1 to 10 to 1 to 5120. A control group of 3 rabbits was given typhoid vaccine 1 cc intramuscularly and the agglutination titre followed through its peak response of 1 to 1280 on the 21st day until the titre had fallen. On the 80th to 95th day, nitrogen mustard (methyl bis β chlorethyl amine) 1 mg per kg (total dose 2 mg) was injected intravenously every 5 days for 4 doses. No increase in the antibody titre similar to the anamnestic reaction was observed. A second control group of 3 rabbits received injections of 1 cc typhoid vaccine intramuscularly for 3 weeks with a resulting increase in antibody titre to 1-5120 in the third week. Nitrogen mustard 1 mg every 4 days was given for 3 doses. There was no summation of titre but a decline to 1-1280 over the following week occurred.

A group of 3 rabbits was given nitrogen mustard at a dose of 0.5 mg per kg weight (1 mg total dose) every 4 days for 9 weeks. On the 25th day, when the lymphocyte count had fallen to 30% of the pretreatment level, typhoid vaccine 1 cc was given intramuscularly. The antibody titre increased only 1-80 3 weeks later, while the controls reached a titre of 1-1280. Another group of 3 rabbits received a similar dose of nitrogen mustard for 7 weeks. On the 18th day, 3 weekly doses of 1 cc typhoid vaccine were initiated. The treated group showed an antibody titre of 1-320 in the third week as compared to a control level of 1-5120.

The antibody response following simultaneous injection of typhoid vaccine and nitrogen mustard was investigated to determine to what extent this cytotoxic material might interfere with the antibody response. Typhoid vaccine 1 cc intramuscularly was given on the first day and nitrogen mustard 0.5 mg

TABLE I.
Typhoid Agglutination Titers Following Intravenous Injection of Nitrogen Mustard.

Group		No. rabbits	Weeks 0	1	2	3	4	5	6	7	11	12	13
1	Control anamnestic	3	0	1/320	1/640	1/1280	1/640	1/320	1/80	1/10	1/10	0	0
			*	*	*	*	‡				8	9	10
2	Control summation	3	0	1/80	1/640	1/5120	1/1280	Died					
							‡						
3	Preantigen and concurrent	3	0	0	0	0*	0	1/40	1/80	1/40	1/10	1/10	
						‡							
4	Preantigen and concurrent	4	1/20	1/20	1/20	1/160*	1/320*	1/80*	1/40				
						‡							
5	Simultaneous antigen and nitrogen mustard	3	0*	1/160	1/160	1/80	1/160	1/80	1/80				

* Typhoid vaccine 1 cc, 500 million bacteria intramuscularly.
† Methyl-bis-β chlorethyl-amine hydrochloride 2 mg intravenously every 5 days, 4 doses.
‡ Nitrogen mustard 1 mg every 4 days.

[2] Dougherty, T. F., and White, A., *Endocr.*, 1944, **35**, 1.

per kg weight (total dose 1 mg) was given every 4 days for 4 weeks. The antibody titre rose to 1-160 as compared to a control titre of 1-1280.

Comment. The studies of Ehrich[1] have shown that the lymphocytes from lymph of a regional node draining an extremity into which antigen has been injected contain an increased titre of antibody on the fourth to sixth day. Dougherty and White[2,3] have shown that corticosterone will produce a lymphopenia and that the decrease in these cells, with their dissolution is associated with a rise in the antibody titre. Since the nitrogen mustards produce a toxic lymphopenia which is apparent in the peripheral blood[4] and lymph node[5] within 4 days, it might be expected that this toxic dissolution would result in the increase of antibody. With this in view, the nitrogen mustard was given after the normal antibody curve had fallen but no rise in titre similar to the anamnestic reaction was seen. No summation of titre occurred when nitrogen mustard was given at the peak of the antibody response.

These findings do not contest the role of the lymphocyte in antibody formation but suggest that the toxicity of nitrogen mustard interferes with the antibody forming mechanism of the lymphocyte. This hypothesis is supported by the suppression of the antibody formation in animals receiving antigen following pretreatment and concurrent treatment with nitrogen mustard. The dosage used in these experiments are 10-fold or more than that given in human therapy and an investigation of the antibody response in the human is now in progress. It is of interest to mention that in the human given a course of 25 mg nitrogen mustard, the leucopenic phase coincides with a period of active regeneration of the hemopoietic system.[6]

Summary. 1. The lymphocytoxic effect of the nitrogen mustards will not produce an anamnestic reaction or summation of the antibody titre in the rabbit.

2. Pretreatment and concurrent administration of nitrogen mustard suppress the antibody response to typhoid antigen.

3 Dougherty, T. F., White, A., and Chase, S. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 28.

4 Jacobson, L. O., Spurr, C. L., Barron, E. S. G., Smith, T. R., Lushbaugh, C., and Dick, G. F., *J. A. M. A.*, 1946, **132**, 263.

5 To be published.

6 Spurr, C. L., Jacobson, L. O., Smith, T. R., and Barron, E. S. G., A.A.A.S. Gibson Island Conf. on Cancer, *Chemotherapy of Tumors,* in press; *Cancer Research,* 1947, **7**, 51.

15764

Chemotherapeutic Action of Streptomycin and of Streptomycin with a Sulfone or Sulfadiazine on Tuberculosis.

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Previously published experiments[1] have shown that streptomycin and promin used together in the treatment of experimental tuberculosis in guinea pigs produced a chemotherapeutic effect greater than the sum of effects from the individual components. Equally good results were later reported from the combined use of streptomycin and another sulfone, sodium salt of 4-amino,4′-galacturonylaminodiphenylsulfone.[2] The supply of streptomycin, however, was then so limited that no experiments could be made

1 Smith, M. I., and McClosky, Wm. T., *Pub. Health Rep.*, 1945, **60**, 1129.

2 Smith, M. I., McClosky, W. T., and Jackson, E. L., *Am. Rev. Tub.*, in press.

with streptomycin alone to appraise fully the value of the combined treatment.

Data have been accumulated on the relative chemotherapeutic value of several sulfone derivatives with the object of finding a compound less toxic, and if possible more effective, than promin or the parent substance 4,4′-diaminodiphenylsulfone (DDS). Studies on this phase of the problem have indicated that the mono *n*-propyl derivative of DDS was much less toxic than promin and at least as effective as the latter.[2] Another derivative of DDS, the mono-succinimide, synthesized in this laboratory by one of us (H.B.), was also found to have a low degree of toxicity and appeared worthy of therapeutic trial with streptomycin. A trial experiment with streptomycin and a sulfonamide also appeared desirable, especially since sulfadiazine had previously given some indication of chemotherapeutic activity in experimental tuberculosis, even if only of a very low order, compared with the sulfones.[3]

Accordingly a series of experiments were set up to determine (a) the chemotherapeutic effectiveness of streptomycin alone at a dose level of 40 mg per kg per day, a dose 2 to 4 times as great as had been used hitherto, (b) the effect of streptomycin at half the above dose in combination with one of the 2 sulfones mentioned earlier or with sulfadiazine.

Experimental. A series of male guinea pigs weighing 300 to 350 g, usually about 325 g, were inoculated intraperitoneally with 1 cc of a homogeneous bacillary suspension in saline containing 0.4 mg moist weight of tubercle bacilli, human strain, H37Rv.* The animals were divided into 6 groups of 20 each and treated as follows:

Group A. Streptomycin, 20 mg† per kg intramuscularly twice daily, 9 a. m. and 4 p. m.

Group B. Streptomycin, 10 mg per kg intramuscularly twice daily, and *n*-propyl derivative of diaminodiphenylsulfone (*n*-propyl),‡ 500 mg per kg per day, orally.

Group C. Streptomycin as in Group B and 250 mg per kg per day of the succinimide derivative of diaminodiphenylsulfone (succinimido)§ orally.

Group D. Streptomycin as in Group B and 500 mg per kg per day of sulfadiazine orally.

Group E. *n*-Propyl as in Group B but no streptomycin.

Group F. Succinimido as in Group C, but no streptomycin.

Group G. Untreated controls.

The sulfones and sulfadiazine were administered by stomach tube in 10% aqueous suspension with 5% gum acacia.

Treatment was begun the day after inoculation and continued for 11 weeks, 5 days a week, with a double dose on the fifth day. Three weeks after treatment was discontinued the survivors were tuberculin tested, using 0.01 mg PPD in 0.1 cc saline intracutaneously. Readings were made 24 and 48 hours later. Eighteen to 20 weeks after infection all the survivors were killed with chloroform, autopsied, and the extent of tuberculous involvement recorded as previously described.[5]

In addition to the 6 foregoing groups a small group (L) of 7 guinea pigs was set aside for treatment, beginning 23 days after infection with 10 mg per kg streptomycin twice daily. Treatment in this group was continued for 11 weeks as in the preceding groups. These animals were killed and autopsy findings recorded as in the others.

Results. Table I gives in detail the extent of tuberculous involvement (tuberculosis index, T.B.I.) in the individual animals in each group at autopsy and also the average for each group. The ratings are all based on a possible maximum of 20. The sign ±

[3] Smith, M. I., Emmart, E. W., and Westfall, B. B., *J. Pharm. and Exp. Therap.*, 1942, **74**, 163.

* Courtesy Mr. W. Steenken, Jr., acting for the Committee on Standard Cultures of the Medical Research Committee of the National Tuberculosis Association.[4]

† 1 mg = 1000 units.

‡ $NH_2 \cdot C_6H_4 \cdot SO_2 \cdot C_6H_4 \cdot NH \cdot C_3H_7$.

§ $NH_2 \cdot C_6H_4 \cdot SO_2 \cdot C_6H_4 \cdot N(CO \cdot CH_2)_2$

[4] A Depot for Standard Cultures of Tubercle Bacilli, *Am. Rev. Tub.*, 1946, **53**, 511.

[5] Smith, M. I., Emmart, E. W., and Stohlman, E. F., *Am. Rev. Tub.*, 1943, **48**, 32.

TABLE I.

The Tuberculin Reaction (PPD) and the Extent of Tuberculous Involvement (Tuberculosis Index, T.B.I.) at Autopsy in the Individual Animals in the Several Groups Treated with Streptomycin Alone or in Combination with Derivatives of Diaminodiphenylsulfone (DDS) or Sulfadiazine. All Animals Inoculated with 0.4 mg H37Rv Intraperitoneally.

	G	A		B		C		D		E	F	L
	Controls	Streptomycin		Streptomycin + *n*-propyl DDS		Streptomycin + succinimido DDS		Streptomycin + sulfadiazine		*n*-Propyl DDS	Succinimido DDS	Streptomycin, 23 days after infection
No.	T.B.I.	PPD	T.B.I.	PPD	T.B.I.	PPD	T.B.I.	PPD	T.B.I.	T.B.I.	T.B.I.	T.B.I.
1	13	+	0	*	*	+	±	+	2	2	9	0
2	6	+	0	+	0	+	1	±	2	2	3	2
3	10	+	±	±	0	+	2	±	1	6	4	2
4	20	+	±	+	0	0	±	+	1	3	11	6
5	5	+	2	+	0	±	2	±	±	1	6	±
6	19	0	1	±	0	+	1	0	0	3	14	2
7	20	0	0	+	0	±	±	0	0	3	2	5
8	12	+	2	±	0	±	0	+	2	1	13	
9	20	+	2	0	1	+	1	0	2	3	14	
10	15	0	0	+	±	+	0	+	±	2	1	
11	9	+	1	+	0	0	1	+	2	6	10	
12	18	±	1	0	0	±	0	+	1	4	11	
13	20	+	2	0	±	+	1	0	0	2	15	
14	11	0	1	±	0	±	0	0	±	9	8	
15	20	+	1	*	*	±	1	+	±	5	2	
16	11	+	0	+	3	±	±	+	0	6	2	
17	16	+	2	±	2	+	±	±	2	12	8	
18	20	±	±	+	1	+	0	0	±	1	10	
19	10	+	2	+	0		±	±	0	2	13	
20	13	+	1	+	1	+	3	+	2	6	3	
Avg	14.4		1.0		0.5		0.8		1.0	3.9	7.9	2.4

* Died accidentally at an early date.

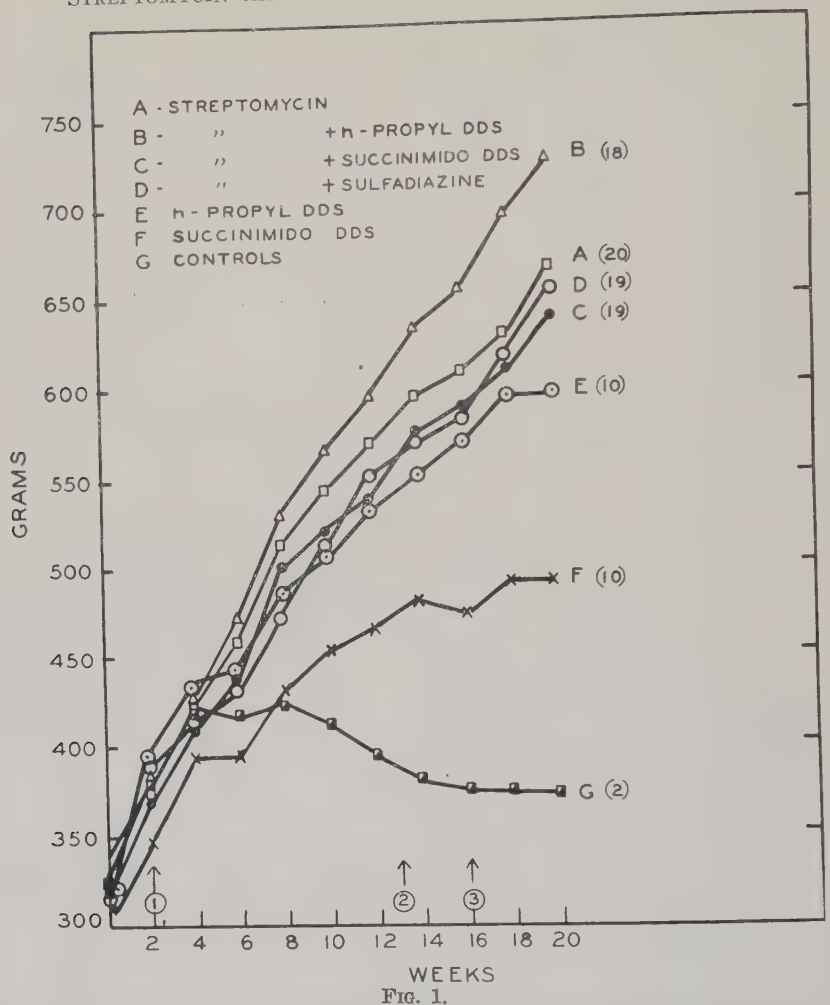

FIG. 1.

Average weight curves of groups of tuberculous guinea pigs treated with streptomycin (A), streptomycin and a derivative of diaminodiphenylsulfone (B and C), streptomycin and sulfadiazine (D), sulfone derivatives alone (E and F). G represents a group of untreated controls. Figures in parentheses indicate number of animals surviving at termination of experiment. First arrow shows time of infection and when treatment was begun; second arrow indicates end of treatment; at third arrow the survivors were tuberculin tested.

For details as to dosage and other pertinent data see Table II.

designates doubtful lesions and has been arbitrarily assigned a numerical value of 0.5. The results of the tuberculin tests for Groups A, B, C, and D are given in the PPD columns of the respective groups.

Group G Controls. Eighteen animals in this group (90%) died with extensive generalized tuberculosis in from 44 to 97 days. The remaining 2 animals were killed at the termination of the experiment, 126 days after infection, and these, also, had advanced tuberculosis of the viscera. The tuberculosis index in the individual animals varied from 5 to 20 (out of a possible maximum of 20) with an average of 14.4.

Group A. All the animals treated with 20 mg per kg streptomycin twice daily survived the experimental period, and made good gains in weight, as shown in curve A, Fig. 1. Though 14 animals in this group gave positive tuberculin reactions, 8 (40%) showed none or only doubtful lesions at necropsy. The remainder had a tuberculosis index of 1 to 2, with an average of 1 for the entire group.

Group B. Two of the animals treated with

streptomycin and the *n*-propyl compound died in 27 and 32 days respectively. At autopsy there were emphysema and congestion of the lungs in one and pulmonary edema in the other. The animals had shown no signs of illness up to the last drug feeding by stomach tube, and death in these 2 animals was regarded as accidental. The remaining 18 animals in this group gained weight normally, survived the full experimental period, and while 10 of them gave positive tuberculin reactions, 13 (72%) showed no gross evidence of tuberculosis at autopsy. The animals in this group showed the highest average gain in weight (curve B, Fig. 1). The average tuberculosis index for the entire group was 0.5.

Groups C and D. The animals treated with streptomycin in combination with the succinimido compound and sulfadiazine respectively compared favorably with the animals in Group A receiving streptomycin alone at twice the dose level. One animal in each of these groups died, at 71 and 104 days respectively, and while pulmonary emphysema was present in both neither showed much evidence of tuberculosis. The average tuberculosis index for Group C was 0.8, and 11 animals (55%) showed no gross evidence of tuberculosis. Ten animals in this group gave positive tuberculin reactions. The average tuberculosis index for Group D was 1.0, with 10 animals (50%) showing no macroscopic lesions, and 9 reacting to tuberculin. The weight curves of the animals of these 2 groups were nearly identical with that of Group A.

Groups E and F. Treatment with the *n*-propyl and succinimido derivatives respectively resulted in the mortality of 50% in each group. However, all of the survivors in Group E were in very good condition, while 4 of the survivors in Group F at the termination of the experiment were losing weight. The average weight curve for Group E was decidedly better than that of Group F. At autopsy all the animals in both groups had definite macroscopic tuberculosis. However, the average tuberculosis index for Group E was 3.9 with a variation of 1 to 12, while

TABLE II.
Summary of All Tests in the Several Groups.

Group and drug, mg/kg/day	G Controls	A Streptomycin 2 × 20	B Streptomycin 2 × 10 + *n*-Propyl DDS 500	C Streptomycin 2 × 10 + succinimido DDS 250	D Streptomycin 2 × 10 + sulfadiazine 500	E *n*-Propyl DDS 500	F Succinimido DDS 250
Mortality % at termination of exp. 130-140 days after infection	90	0	0	5	5	50	50
Avg T.B. index computed on basis of 100 for controls	100	6.9	3.5	5.5	6.9	26.9	54.5
Percent with no or doubtful lesions (T.B. rating of 0 or ±)	0	40	72	55	50	0	0
Chemotherapeutic effectiveness (ratio of T.B. index of controls and treated groups)		14.4	28.6	18.2	14.5	3.7	1.8
Avg wt gains, g	53	342	402	332	351	278	207
Wt of spleen, g							
Range	1.0-12.0	0.7-1.8	0.7-1.6	0.7-1.4	0.6-1.2	0.9-7.0	0.7-9.9
Avg	5.4	1.0	1.2	0.9	0.9	2.8	2.5

TABLE III.
Blood Levels. Average of 3 to 4 Animals.

Drug	Daily dose, g/kg	Mg %, hr after administration		
		3	5	19-24
n-Propyl DDS	0.25	1.5	1.5	0.8
	0.50	2.0	2.1	1.2
	2.00	3.4	3.6	3.8
Succinimido DDS	0.25	1.8	0.7	0.5
	2.00	1.6	1.5	1.6

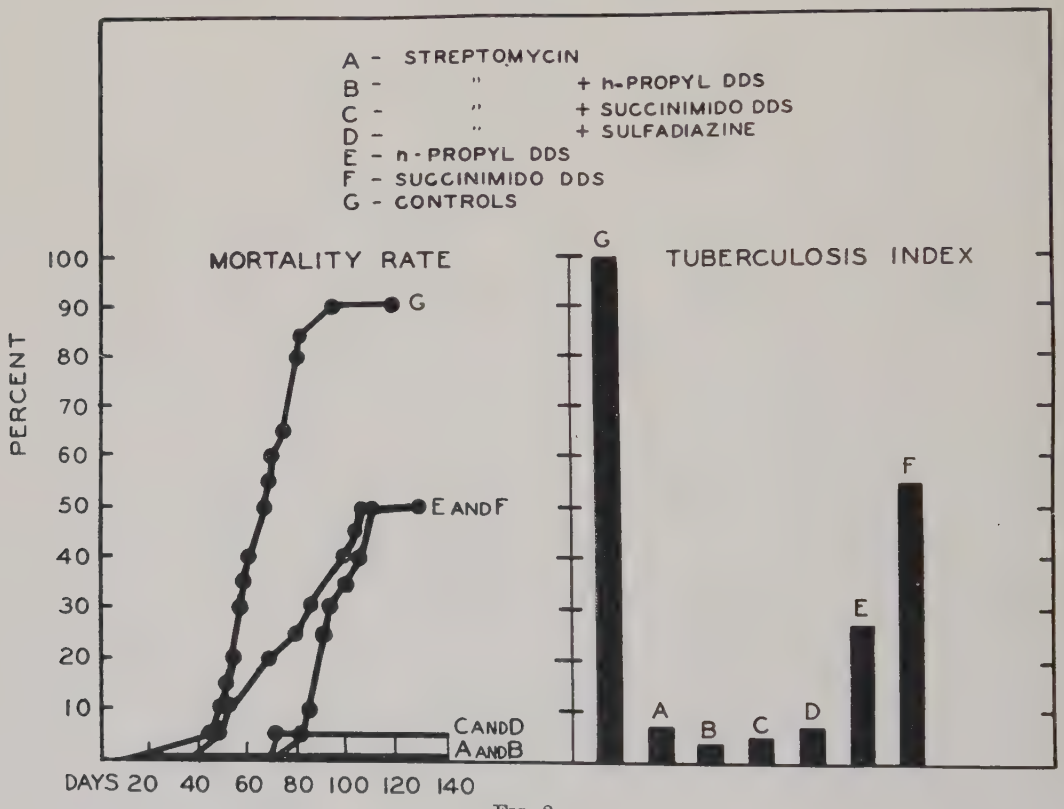

FIG. 2.
Mortality rate and average extent of tuberculous involvement (tuberculosis index) in groups of tuberculous guinea pigs treated as indicated in legend of Fig. 1.

in Group F the average index was 7.9 with a variation of 1 to 15. This evidence appears to indicate that while neither drug eradicated the disease the *n*-propyl derivative had the much greater suppressive effect of the 2.

Group L. The animals in this group in which treatment was begun 23 days after infection all survived the experimental period, with marked improvement in their weight curve after treatment was instituted, and necropsy at the termination of the experiment showed an average tuberculosis index of 2.4 with a variation of 0 to 6. Two of the 7 animals showed no gross evidence of the disease.

Tissues from each of the 7 animals in this group, together with tissues from 5 animals of the control group, were submitted to Dr. R. D. Lillie of the Laboratory of Pathology for microscopic examination. These included liver, spleen, lungs, kidneys, testes, omentum, and diaphragm. His re-

port is summarized as follows:

Of the 5 controls one showed chronic progressive tuberculosis, 2 had subacute progressive tuberculosis, and 2 showed active uninhibited tuberculosis. Two of the treated animals (L 1 and L 5) had no tuberculosis, the latter showing subacute bronchopneumonia. The others presented the following:

L 2. Solitary tubercle of spleen, relatively inactive.

L 3. Old lymphadenoid tuberculosis, relatively inactive.

L 4. Inactive old tuberculosis with calcification and fibrosis in the lungs and lymph nodes. Chronic bronchopneumonia and bronchiectasis.

L 6. Old lymphadenoid and peritesticular tuberculosis with some calcification and fibrosis and chronic interstitial pneumonia.

L 7. Minimal chronic tuberculosis of the lungs, spleen and mesenteric lymphnodes with bronchopneumonia and bronchiectasis.

These findings agree well with the data given in Table I, and recorded independently on the basis of gross observation.

In Table II are summarized the results of the entire experiment. Comparison of the relative values of the different forms of treatment, based on the most important criteria, namely mortality per cent, average tuberculosis index, and incidence of freedom from disease as judged by macroscopic examination, all point to the superiority of combined treatment with streptomycin and the *n*-propyl derivative of diaminodiphenylsulfone. The average gains in weight of the animals in the several groups as shown in Table II and Fig. 1 also point to the same conclusion.

Of the 2 sulfones presented in this study the data in Tables I and II and in Fig. 1 and 2 indicate that the *n*-propyl derivative has the greater suppressing effect on the disease. Both compounds have a low degree of toxicity and are characterized by poor absorbability, as shown in Table III. It is to be noted that the succinimido compound was used in the therapeutic tests at half the dose level of the *n*-propyl derivative, but it is doubtful whether it would have been more effective at the higher dose level, since in normal animals little more of this drug was absorbed when administered in daily doses of 2.0 g per kg than when given in doses of 0.25 g per kg.

Discussion. Viewing the results of the present study against the background of those previously reported it is becoming increasingly evident that streptomycin is by far the most effective chemotherapeutic agent in experimental tuberculosis. Working under fairly well standardized conditions, with a rather heavy infection giving a mortality of from 65 to 95% of the controls in 90 to 100 days, treatment with streptomycin at a dose level of 10 to 15 mg per kg per day gave a chemotherapeutic effectiveness of 5.2, no mortality within the experimental period, and absence of macroscopic tuberculosis in 15% of the animals.[1] When the daily dose of streptomycin was increased to 40 mg per day in the present series under very similar conditions a chemotherapeutic effectiveness of 14.4 was obtained, no mortality, and 40% of the animals appeared to be free from lesions. This latter dose of streptomycin is approximately 1/10 of the LD_{50}.[1] It is evident therefore that the response is roughly proportional to dosage, and it is not impossible that further improvement may be had with increasing dosage. It is to be remembered however, that in all cases treatment was begun the day after infection and continued for 60 to 80 days. The picture is not as bright however, when treatment is delayed, as it was done in the present study, Group L, where treatment with streptomycin was not begun until 23 days after infection in which case 5 of the 7 animals had slight to moderate degrees of tuberculosis and a chemotherapeutic effectiveness for the group of only 6.0 (14.4/2.4) was obtained.

Summarizing the present and previous results on the action of streptomycin and the sulfones used individually or in combination it appears that there is good evidence of potentiation every time combined therapy has been tried. Obviously the better the sulfone the more impressive the effect. For convenience the essential data of the present study and of the 2 previous publications[1,2] have been put together in Table IV. Expressing the value of a given treatment

TABLE IV.
Summary of Essential Data on the Effects of Streptomycin Used Alone or in Combination with a Derivative of Diaminodiphenylsulfone in Experimental Tuberculosis.

Streptomycin mg/kg/day	Sulfone derivative	Chemotherapeutic effectiveness	Mortality %	Free from lesions, %	Mortality, % of controls	Duration of exper., days	Reference
10-15	0	5.2	0	15	65	110	1
0	Promin	2.4	15	5	65	110	1
10-15	,,	20.0	0	65	65	110	1
0	Galacturonyl	2.7	70	5	95	103	2
20	,,	27.0	5	75	95	103	2
40	0	14.4	0	40	90	140	present
0	*n*-Propyl	3.7	50	0	90	,,	,,
20	,,	28.6	0	72	90	,,	,,
0	Succinimido	1.8	50	0	90	,,	,,
20	,,	18.2	5	55	90	,,	,,
20	Sulfadiazine	14.5	5	50	90	,,	,,
20*	0	6.0	0	28	90		

* Treatment delayed for 3 weeks after infection.

in terms of "chemotherapeutic effectiveness" (the ratio of the average extent of tuberculous involvement in the control and treated groups) it is evident that the efficacy of the combination is greater in every instance than the sum of effects from the individual components. Also the highest percentage incidence of animals free from gross lesions has been realized in the experimental groups treated with the combination, though a very substantial percentage appeared to be free from the disease in the group treated with streptomycin alone at the highest dosage level it has been employed.

It is interesting that sulfadiazine appears to have contributed, if anything, less than any of the sulfones in the combination with streptomycin. The effectiveness of sulfadiazine in the treatment of experimental tuberculosis is also slight compared with the sulfones.[3,6]

Little can be said at this time concerning the possible mechanisms that may be involved in the mutual potentiation of streptomycin and the sulfones. Our knowledge of the mechanism of antibacterial action of either streptomycin or of the sulfones is fragmentary and inadequate. However, evidence has been accumulating to indicate that bacteria can acquire a high degree of resistance against the antibiotics including streptomycin.[7-9] Youmans and Williston[10] have also shown recently that the tubercle bacillus may acquire a resistance to streptomycin, that streptomycin resistant strains are equally virulent in mice, and the animals so infected are refractory to treatment with streptomycin. We had previously shown in this laboratory that prolonged cultivation of

6 Smith, C. R., and Oechsli, F. W., *Am. Rev. Tub.*, 1945, **52**, 86.

7 Miller, C. P., and Bohnhoff, M., *J. Am. Med. Assn.*, 1946, **130**, 485.

8 Graessle, O. E., and Frost, B. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 171.

9 Finland, M., Murray, R., Harris, H. W., Kilham, L., and Meads, M., *J. Am. Med. Assn.*, 1946, **132**, 16.

10 Youmans, G. P., and Williston, E. H., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 131.

the tubercle bacillus in a medium containing low concentrations of promin resulted in attenuation of virulence.[11] It is possible therefore that the beneficial effect of the combined action of the 2 chemotherapeutic agents consists in eliminating or attenuating strains which might acquire a resistance to the antibiotic.

Summary and Conclusions. The chemotherapeutic action of streptomycin used alone or in combination with one of two sulfones or sulfadiazine was studied in experimental tuberculosis in guinea pigs.

Evidence has been obtained to indicate that the therapeutic effect from combined treatment is greater than the sum of effects from the individual components.

The chemotherapeutic effectiveness (ratio of extent of tuberculous involvement in a group of untreated controls and treated groups) in a group of guinea pigs treated with 40 mg (40,000 units) streptomycin per kg of body weight daily was 14.4. Previously treatment with 10 to 15 mg per kg per day under similar conditions gave a chemotherapeutic effectiveness of 5.2.

The chemotherapeutic effectiveness of combined therapy with 20 mg streptomycin per kg per day plus 500 mg 4-amino-4′-propylaminodiphenylsulfone per kg per day was 28.6. The chemotherapeutic effectiveness of the sulfone alone was 3.7.

Similar though less marked potentiation was obtained in combined therapy with streptomycin and 4-amino-4′-succinimidodiphenylsulfone. By itself this latter sulfone was less effective than the *n*-propyl derivative.

Combined therapy with streptomycin and sulfadiazine, a substance of doubtful efficacy in experimental tuberculosis, gave inconclusive evidence of potentiation.

11 Emmart, E. W., and Smith, M. I., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 320.

15765

Further Studies on the Testing of Sterility of Concentrated Streptomycin Solutions.

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The inactivation of streptomycin with semicarbazide, as an aid in testing the sterility of concentrated solutions of streptomycin, was proposed in an earlier paper.[1] A similar method of sterility testing involving the use of hydroxylamine has been described in tentative minimum specifications for streptomycin issued by the Food and Drug Administration.[2] As is shown in Table I, semicarbazide, as compared to hydroxylamine mol for mol, has the advantage for such sterility test procedures in that the bacteriostatic action of the former is less than that of the latter for most organisms tested.* If instead of thioglycolate broth, which was used in the present tests, tryptone broth is used, the differences in the bacteriostatic action of the two compounds are even more marked in favor of semicarbazide.[1] The same is true of effects on bacteria in aqueous streptomycin solutions in which the antibacterial power of the carbonyl reagent would be exerted in the suggested sterility tests.

However, recent experiences lead to the

1 Rake, G., and Donovick, R., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 31.

2 *Tentative Minimum Specifications for Streptomycin,* Food and Drug Admin., June 28, 1946.

* Amongst the organisms tested were a number of strains kindly made available to us by Dr. W. A. Randall of the Food and Drug Administration and said to be less susceptible to hydroxylamine than to semicarbazide.

TABLE I.
Comparative Bacteriostatic Action of Semicarbazide-HCl and Hydroxylamine-HCl Against Various Test Organisms in Thioglycolate Broth.

Test organism	M.I.C.* Semicarbazide HCl mg/ml	Semicarbazide HCl millimols/L	Hydroxylamine HCl mg/ml	Hydroxylamine HCl millimols/L	Mol ratio SC/HA
E. coli SR†	0.60	5.4	0.30	4.3	1.25
K. pneumoniae (A.T.C.C. No. 9997)	0.21	1.9	0.17	2.4	0.79
No. 2411‡	0.15	1.3	0.044	0.63	2.06
No. 2435‡	0.14	1.3	0.039	0.56	2.32
B. brevis§	0.71	6.4	0.18	2.6	2.46
B. circularis§	0.79	7.1	0.20	2.9	2.44
B. subtilis (Peoria)§	0.76	6.8	0.23	3.3	2.06
Pseudomonas aeruginosa§	0.30	2.7	0.17	2.4	1.12
Aerobacter aerogenes§	0.37	3.3	0.25	3.6	0.92
B. subtilis (Merck)§	0.35	3.1	0.13	1.9	1.63
Staphylococcus aureus (Osgood)§	1.16	10.4	0.14	2.0	5.20

* Minimum inhibiting concentration.

† Strain of *E. coli* isolated from solution containing *ca.* 100,000 units streptomycin/ml.

‡ Psychrophilic organisms isolated from solution containing *ca.* 100,000 units streptomycin/ml.

§ These strains of bacteria were obtained from the Food and Drug Administration through the kindness of Dr. William A. Randall.

conclusion that the use of either carbonyl reagent can result in false negative results as to the presence of contaminants in concentrated streptomycin solutions. These findings were anticipated somewhat in the earlier paper[1] when it was pointed out that, since carbonyl reagents *per se* are bacteriostatic substances, the procedure suggested (for inactivation of and sterility testing of streptomycin) was valid only to the extent that viable organisms in streptomycin solutions were able to withstand the action of carbonyl reagents.

In recent months it has been noted that certain solutions of streptomycin, containing *ca.* 100,000 units per ml, on standing at room temperature or at 5°C, became turbid with what appeared to be bacterial growth. While sterility tests according to the procedure described[1] failed to show any growth, simple streaks of these original solutions on yeast beef agar plates yielded one, and in some cases 2, strains of bacteria. One of these was a strain of *Escherichia coli* and 2 others (No. 2411 and No. 2435, possibly identical, although isolated from separate sources) were Gram negative, psychrophilic rods which grew readily at 5° to 25°C but poorly at 37°C.

Pure cultures of these 3 organisms were inoculated into streptomycin solutions containing *ca.* 30,000 units per ml. The inoculated solutions were divided into several portions, some of which were treated with semicarbazide and potassium acetate as described earlier,[1] while the remainder served as controls. Although the organisms could be readily recovered from the untreated streptomycin solutions, they could not be reisolated from the solutions treated with semicarbazide. Similarly failure resulted from subsequent tests with equivalent amounts of hydroxylamine as the inactivating agent, and, as shown in Table I, all 3 organisms were more sensitive to hydroxylamine than to semicarbazide.

Thus, whereas the use of carbonyl reagents had aided in the demonstration of the presence in streptomycin solutions of certain spore formers,[1] they now played a detrimental role. There still remains a need for a non-germicidal agent which will inactivate streptomycin. Van Dolah and Christenson[3] have recently suggested the use of certain oxidizing and reducing agents, such as potassium permanganate and potassium periodate, for the inactivation of streptomycin. However, here again it is likely that, in higher concentrations required for a sterility test, some bacterial destruction would be caused by the reagent before the reaction had gone to completion and before the streptomycin-reagent mixture could be added to test culture media.

[3] Van Dolah, R. W., and Christenson, G. L., *Arch. Biochem.*, 1947, **12**, 7.

Further consideration apparently should also be given to the choice of culture medium and the temperature of incubation in sterility testing of streptomycin solutions. Broth containing sodium thioglycolate, because of the ability of the latter to interfere with the action of many antibacterial substances,[4] is widely used for sterility testing of biological materials. The extent to which this broth interferes with the action of streptomycin[5] led to its use in a proposed sterility test.[1] In attempts, however, to isolate the streptomycin-resistant *E. coli* strain, referred to above, from inactivated mixtures of streptomycin and carbonyl reagents, it was found that control cultures died out in one week at 37°C. On further examination it appeared that this organism could be carried in thioglycolate broth if daily subcultures were made, but if transfers were attempted after one week's incubation no growth ensued. It was found that this organism could be recovered from this broth through 6 days of incubation but not after the 7th day. Controls in yeast beef broth yielded positive subcultures throughout at least 8 days.

In the sterility testing of many biological materials it is not an uncommon practice to inoculate the material under question into thioglycolate broth, incubate for one week, and then subculture if there is any doubt whether growth has occurred. Should this be done with a material containing a contaminant such as the *E. coli* strain used here, the subcultures would yield no growth. In this connection we have found that the 2 psychrophils mentioned above do not grow well in thioglycolate broth, but multiply readily in yeast beef broth.

The occurrence of 2 psychrophilic organisms as contaminants in several highly concentrated streptomycin preparations which we have studied demonstrates also the need for incubating streptomycin sterility tests at more than one temperature. We have not yet found any thermophilic organisms occurring as natural contaminants in streptomycin solutions, but this possibility should not be overlooked.

Summary. 1. Further studies with semicarbazide and hydroxylamine confirm earlier findings that for most of the bacterial species tested the former substance mol for mol is less bacteriostatic than the latter. 2. The use of either carbonyl reagent in sterility tests of concentrated streptomycin solutions, however, may lead to false negative findings. 3. Three strains of microörganisms have been isolated from streptomycin solutions which failed to grow out from similar material inactivated with either carbonyl reagent. 4. These organisms isolated from the streptomycin solutions grow poorly in thioglycolate broth, and one species was found to die out in this broth on incubation prolonged beyond 6 days. 5. The demonstration of psychrophilic organisms in streptomycin solutions suggests the need for incubating such sterility tests at more than one temperature.

[4] a. Eagle, H. J., *Pharmacol.*, 1939, **66**, 436; b. Fildes, P., *Brit. J. Exp. Path.*, 1940, **21**, 67; c. Chow, B. F., and McKee, C. M., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 175; d. Cavalito, C. J., Bailey, J. H., Haskell, T. H., McCormick, J. R., and Warner, W. F., *J. Bact.*, 1945, **50**, 61; e. Geiger, W. B., and Conn, J. E., *J. Am. Chem. Soc.*, 1946, **67**, 112.

[5] Donovick, R., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 224.